ISSN 2221-6421 (Print), ISSN 2223-2567 (Online)
Coden: PJSIC6 64(1) 01-108 (2021)

Pakistan Journal of Scientific and Industrial Research

Series B: Biological Sciences

Vol. 64, No.1, January-April, 2021

(for on-line access please visit web-site http://www.pjsir.org)

Published by
Scientific Information Centre
Pakistan Council of Scientific and Industrial Research
Karachi, Pakistan

Pakistan Journal of Scientific and Industrial Research

Series B: Biological Sciences (Vol.64, Issue 1, 2021)

Pakistan Journal of Scientific and Industrial Research is published triannually i.e:

Series B: Biological Sciences [**ISSN 2221-6421 (Print); ISSN 2223-2567 (Online)]** (appearing as issues of January-April, May-August and September-December).

This Journal is indexed/abstracted in Zoological Record, BIOSIS Preview, NCBI, Pub Med, Scimago, Research Gate, Clarivate Analytics, CABI, Elsevier's Scopus.

Subscription rates (including handling and Air Mail postage): ***Local:*** Rs. 1500 per volume, single issue Rs. 500
Foreign: US$ 450 per volume, single issue US$ 75

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Composed by: Syed Asif Ali, Imdadullah Khan and Kamran Ahmed; Graphics by: Mansoor Ghani; Webmaster: Mobeen Ali

Printed and Published by: PCSIR Scientific Information Centre, PCSIR Laboratories Campus, Shahrah-e-Dr. Salimuzzaman Siddiqui, Karachi-75280, Pakistan.

Editorial Address

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Pakistan Journal of Scientific and Industrial Research, PCSIR Scientific Information Centre,
PCSIR Laboratories Campus, Shahrah-e-Dr. Salimuzzaman Siddiqui, Karachi-75280, Pakistan
Tel: 92-21-99261914-99261916, 99261949-99261917; Web: http://www.pjsir.org, E-mail: info@pjsir.org

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Pakistan Journal of Scientific and Industrial Research
Series B: Biological Sciences
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Review

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Nutritional Assessment and Biological Activity of *Moringa oleifera*

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(received October 15, 2018; revised March 11, 2019; accepted March 12, 2018)

Abstract. *Moringa oleifera* medicinal plant is used for medicinal purposes for the treatment of different types of human diseases. The fresh roots, stems, leaves, flowers and seeds have been selected for such medicinal purposes. The findings of present study indicated that an appreciable amount of proximate composition and phytochemical had been confirmed. The leaves and seeds for this study showed significant phytochemical sources including phenolic compound, flavonoid, tannin alkaloid and antioxidant content, whereas the most sensitive minerals composition sources including Ca, Fe, Mg, Na, K, antibacterial activity including the *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aurous* were found to be the most sensitive, while *Klebsiella pneumonia* least sensitive in this study. The growth of *Escherichia coli* is mostly inhibited by all plants components. Whereas, flowers and leaves showed good inhibition zone against *Escherichia coli*. The flowers of *Maringa oleifera* possessed antibacterial protein and peptide which showed highly significant against *Staphylococcus aurous, Escherichia coli, Klebsiella pneumonia, Pseudomonas, proteus* and *enterobacter.* The research will be supported for nutrition and sources of new drugs for the treatment of the different types of diseases.

Keywords: nutritional, antioxidant, antibacterial protein, medicinal plants

Introduction

Moringa oleifera Lam medicinal plant which belongs to Moringaceae family. It is distributed in the Himalayan foothills from north-eastern Pakistan to north-west Bengal, India, Afghanistan, Srilanka, South and West Asia and Africa. All parts of the plant were used for the treatment of various diseases forms. The root is used to treat different forms of diseases such as, fever, epilepsy, asthma, headache, gout, diarrhoea, hysteria, flatulence, scurvy, low back and kidney pain, flowers used for throat infections, hysteria and rheumatism as well as tonic abortion, seeds for tumour, ulcer, rheumatism and arthritis, and the leaves for bacterial infection, antioxidant, urinary tract infection and diarrhoea, HIV-AIDS, headache, hypertensive, lactating enhancer, fever, hepatic, ulcer, tumour, dysentery, catarrh and scurvy (Chaudhary and Chaurasia, 2017). The leaves of *Moringa oleifera* are also beneficial for the treatment of pneumonia, malaria, hyperglycaemia, diarrhoea, skin disease, flu, anticancer, heart burn antimicrobial, syphilis, dyslipidemia, scurvy, headache, antibiotic, anti-atherosclerotic, antidiabetic, neuroprotectant reduce cholesterol and blood pressure. The seeds for the treatment of chrohn's disease, gout, hyperthyroidism, epilepsy, antiherpes simplex virus arthritis, sexually transmitted disease, cramp, antiinflammatory and antimicrobial agents studied by (Lakshmipriya *et al*., 2016). In the ancient world, *Moringa oleifera* was extremely valued. It is mentioned that leaves and fruits of *Moringa* in their diet to maintain healthy skin and mental alertness in the history dates back to 150 B.C. *Moringa* leaf extract was fed of the ancient Maurian soldiers of India in the warfront. *Moringa* is fabulous food tree with remarkable sources of nutrients including calcium, iron, vitamin C and vitamin A. It is described that the leaves of *Moringa oleifera* contain vitamin A ten times more than carrot, vitamin C seven times more than orange, seventeen times calcium than milk, iron 25 times more than spinach, potassium 15 more than banana and also contain vitamin B complex, magnesium, zinc, copper, manganese, chromium and phosphorus (Bhupendra and Chase, 2015). Antifungal, anti-inflammatory, antimicrobial, antifertility, anti-atherosclerotic, relieving pain, diuretic, regulating hypothyroidism, central nervous system depressant, asthma, hyperglycaemia, pneumonia, malaria, scurvy, diarrhoea dyslidemia, reduce blood pressure, reduce cholesterol, anticancer, antidiabetic, neuroprotection agent, skin disease, headache, bronchitis, eye and ear infection, heartburn, flu and syphilis have been cured by *Moringa oleifera* (Udikala *et al*., 2017;

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Rockwood *et al*., 2013; Mbikay, 2012). The all parts plant including roots, stems, leaves, flowers, fruits (Pod) and seeds are being used in the different types of diseases such as treatment of inflammation, hepatorenal disorders, infectious diseases, cardiovascular and gastrointestinal disorders studied by (Nath *et al*., 2015). The leaves of *Moringa oleifera* have possessed more vitamin A than carrots, and more potassium than bananas, more iron than spinach more calcium than milk, more vitamin C than oranges and protein quality equal to as milk and eggs (Sujatha and Patel, 2017).

Materials and Methods

Sample preparation. The fresh root, stem, leave, flower, seed that were being selected and collected from district Kamber, Sindh, Pakistan, had been dried under shade for about 15-30 days and ground. Further, powdered plants materials were made after dryness and stored in air tight bags for further analysis.

Estimation of phytochemical and biological activity methods. ***Proximate analysis*****.** Total ash and moisture content were identified by oven/muffle furnace. Phytochemicals were screened studied by (Lanjwani *et al*., 2015). Carbohydrates by Anthrone method (Rawat *et al*., 2012), Protein by lowery method (Naidu *et al*., 2013).

Phytochemical analysis**.** Total phenolic contents were determined by Folin Ciocalteu method (Maurya and Singh, 2010), flavonoid by aluminium chloride method (Damodar *et al*., 2011), tannin by modified Prussian method (Sathishkumar and Baskar, 2015), total alkaloids by Dragendorff's method by (Sonal *et al*., 2011).

Minerals analysis**.** The plant samples were acid digested for mineral determination detected by (Lanjwani *et al*., 2016). Minerals were identified by atomic absorption spectrometry (Perkin Elmer AA 800).

Biological analysis**.** The content of antioxidant identified by iron reduction method (Patel *et al*., 2010), antimicrobial content by Agar-Well Diffusion Assay (Bonjar, 2014).

Antibacterial protein and peptide. ***Preparation of plant extracts*****,** 10 g of plant powdered sample was taken and 100 mL of distilled water was added and kept on shaking water bath at room temperature for 24 h and then filtered through Whatman filter paper no 1. Filtrate was treated with 20 % trichloroacetic acid. Solution was stand at room temperature for one hour and centrifuged at 6000 rpm for 15 m. Precipitate is collected and solution was checked by 20% trichloroacetic acid for further precipitation. Precipitate was washed with acetone and dried. Precipitate was fully dissolved in distilled water than filtered was used for antibacterial protein 20 µL every sample are used.

Results and Discussion

The results of present study showed that an appreciable amount of phytochemicals indicated that the aqueous extract is the best solvent for extraction including alkaloid, amino acids, phenolic compounds, tannins and flavonoids, whereas methanol extracts is the best solvent for saponins, proteins, carbohydrates, glycosides, terpenoids, steroids, fats and oils. The presence of phytochemicals has diversed medicinal properties including alkaloids for antimalarial, antispasmodic, analgesic and diuretic activity. Terpenoid for anticancer, antiviral, antibacterial, anthelmintic, antimalarial and anti-inflammatory and inhibition of cholesterol synthesis and also possess insecticidal activities. Saponin for antiviral, anti-inflammatory defense of plant and cholesterol reducing activity. Glycosides for antibacterial and antifungal properties. Phenols and flavonoid for antioxidant, antibacterial and antiallergic, etc. (Padalia and Chanda, 2015; Moteriya *et al*., 2015).

Proximate composition is shown in the Table 2, the high ash values obtained in this analysis, which is good sources of inorganic minerals. It is positive indication that high ash indicates high deposition of minerals it is good sign because ash is composed of minerals. Such variations may be occurred due to different areas of research which is impacted by various factors including climates geographical conditions. For tropical climates high moisture will be suitable in replacing loss of water from the body. The root of plants showed good sources of carbohydrate, whereas the highest % of protein was observed in the seeds. The building block of cell and body is protein. The leaves of *Moringa oleifera* have been used in eating in African countries including Ethiopia, Ghana, east Africa, Nigeria and Malawi. *Moringa* tree is also cultivated for foods, nutritional and medicinal purposes (Gomashe *et al*., 2014). The present result of protein and moisture were compared which was similar to reported (Yameogo *et al*., 2011; Manzoor *et al*., 2007). The protein and carbohydrate were investigated in the seeds, while similar finding reported by (Oliveira *et al*., 1999). The carbohydrates and ash contents in the leaves, flowers and seeds were

Table 1. Phytochemical screening of the different parts of *Moringa oleifera*

Phytochemical	Root		Stem		Leave		Flower		Seed	
	MET	AQ	MET	AQ	MET	AQ	MET	AQ	MET	AQ
Alkaloids	-	-	-	-	+	-	-	-	-	++
Phenolic compounds	+	+++	+++	+++	+	+++	+	+++	+	+++
Tannins	+++	+++	+++	+++	+	+++	+++	+++	+	+++
Flavonoids	-	++	-	-	+	-	-	++	++	+
Saponins	++	+	+	+	+	+	++	++	+++	+++
Amino Acids	+++	+++	-	+++	-	+++	-	+++	+++	+++
Protein	+	+	+++	+	+	+	+	+	+	+
Carbohydrates	+++	+	+++	+	+	+++	++	+++	+++	+++
Glycosides	+	+	+	-	-	+++	+	-	+	-
Steroids and triterpenoids	+++	++	+++	-	+++	+++	+++	+++	+++	+
Fat and oils	+	+	+	+	+	+	++	+	+++	+
Vitamin C	-	-	-		-	+	-	-	-	-

Note: (+++) = Appreciable; (++) = Moderate; (+) = Trace amount; (-) = absent; (MET) = Methanol; (AQ) = Aqueous.

Table 2. Proximate composition (%) of *Moringa oleifera*

Parts of plants	Moisture	Ash	Carbohydrate	Protein
Roots	83	21	30.7	6.8
Stems	82	13.9	28.3	3.6
Leaves	77	20	28	10.1
Flowers	78.2	22	27.3	5.3
Seeds	24	20	19.8	36.7

being compared to the reported studies indicated in range (Sanchez-Machado *et al*., 2010).

Phytochemical and antioxidant content are shown in the Table 3. The leaves and seeds for this study showed appreciable sources of phytochemical including phenolic compounds, flavonoids, alkaloids and antioxidant content. This is investigated that phytochemical have adversed beneficial effects in the humans. For example, phenolic compounds and tannins, flavonoids having hypocholesterolemic, hypoglycaemic, anti-inflammatory, antioxidant, anticancer, antihypertensive properties (Oluwole *et al*., 2013). In the present result, the total phenolic compounds, flavonoid and alkaloid were lower and tannin higher than reported by (Adeyemi *et al*., 2014).

Table 3. Phytochemical and antioxidant content (mg/g) of *Moringa oleifera*

Parts of plants	Phenolic compound	Flavonoid	Tannin	Alkaloid	Antioxidant
Roots	112	20	64	0	62
Stems	221	51	65	0	18
Leaves	230	101	64	9.8	45
Flowers	153	37	65	0	55
Seeds	154	84	62	150	48

Mineral composition is shown in the Table 4. It is observed that appreciable sources of principle essential macrominerals including Ca, Fe, Mg, Na, and K. The leaves showed top nutritional values. The present results of Ca, Na, Fe and Zn were compared with previously reported including calcium and iron were investigated near about reported (Padalia and Chanda, 2015). The present results of Mg, Fe are higher and Ca, Na, K and Mn lower than reported by (Aslam *et al*., 2005). Zn and Ni contents were investigated to be higher in degree, while Cr, Cd, Lead were lower than reported by (Limmatvapirat *et al*., 2013). The concentrations of

Table 4. Mineral composition (mg/Kg) of *Moringa oleifera*

Nutrients	Root	Stem	Leave	Flower	Seed
Calcium	2400	4000	16000	2000	1760
Iron	35.12	312.5	625.5	460.9	10.96
Potassium	1204.8	1212.8	1312.8	1101.6	1200
Magnesium	864.2	903.1	562.9	679.6	650.5
Sodium	1768	1136	1680	1300	1600
Zinc	2.2	15.2	17.6	14.6	26
Manganese	45.04	BDL	BDL	BDL	BDL
Cobalt	BDL	25.7	BDL	BDL	BDL
Lead	0.6	5.7	10.8	15.2	BDL
Copper	BDL	BDL	BDL	19.1	4.8
Chromium	BDL	BDL	BDL	0.8	BDL
Nickel	1.7	2.7	3.1	1.5	2 .8
Cadmium	BDL	BDL	BDL	BDL	BDL

Note: (BDL) Below Detection Limit.

cadmium have been indicated below detection limit which is toxic highly even at concentration low. Amount of concentration of lead and cobalt were recorded around permissible limit in the all parts. It is great advantage to be the consumers because of lead is highly toxic. *Moringa* is fabulous food tree with remarkable sources of nutrients including calcium, iron, vitamin C and vitamin A. It is described that leaves of *Moringa oleifera* contain vitamin A ten times more than carrot, vitamin C is seven times more than orange and seventeen times of calcium than milk, iron 25 times more than spinach, potassium 15 more than banana and also contain vitamin B complex, magnesium, zinc, copper, manganese, chromium and phosphorus (Bhupendra and Chase, 2015). Calcium is required for maintaining and formation of bone and teeth, blood clotting and muscle contraction, while potassium is most essential for regulation of water and electrolyte balance, nerve action, muscle function, acid balance in the body. Potassium cause muscle paralysis (Okiki *et al*., 2015).

Antibacterial activity results are summarized in the Fig. 1. Antibacterial activity including the *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aurous* were found to be the most sensitive, while *Klebsiella pneumonia* least sensitive in the present study. The growth of *Escherichia coli* is inhibited by the mostly all parts of plants, whereas flowers and leaves showed good inhibition zone against *Escherichia coli*. It is highly active against *Escherichia coli* and *Bacillus cereus* which may be possessed important beneficial chemical including proteins, phenolic compounds, terpenoids, flavonoids and glycosides. 26 mm inhibition zone was measured by the leaves against *Escherichia coli* that were investigated, while similar in finding 23 mm inhibition zone was reported in (Abalaka *et al*., 2012).

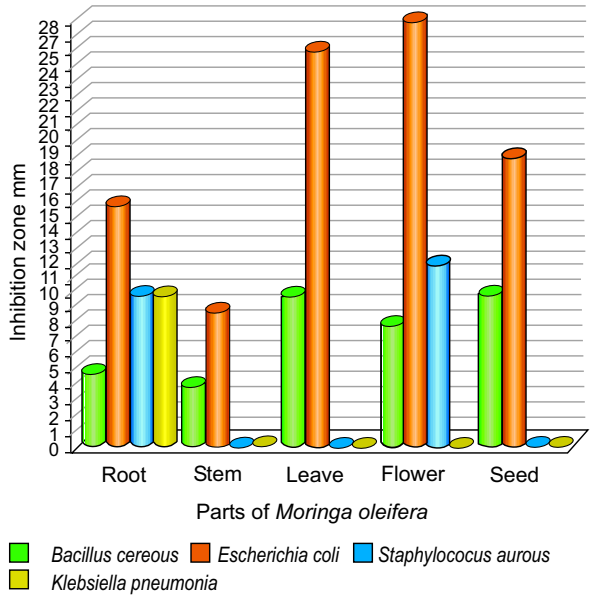

Fig. 1. Antibacterial analysis of methanol extract of *Moringa oleifera*.

Antibacterial proteins and peptides results have been showed in the Table 5. The flower of *Maringa oleifera* was possessed antibacterial proteins and peptides which showed highly significant against *Staphylococcus aurous, Escherichia coli, Klebsiella pneumonia, Pseudomonas, Proteus* and *Enterobacter*. It has been investigated that the flowers can be used to discovered new natural products in the forms of antimicrobial proteins and peptides. Among them proteins and peptides were recently observed as antimicrobial activities. The proteins and peptides are identified as important components of the innate defense system of fungi, bacteria, insects, plants and animals. Most of these defence proteins have multi tasked activities. The several peptides have capabilities to inhibit gram negative and positive bacteria were reported (Reddy *et al*., 2004).

Table 5. Antibacterial protein and peptide from flowers of *Moringa oleifera*

Bacteria	Diameter of zone of inhibition mm
Staphylococcus aurous	15
Pseudomonas	14
Escherichia coli	8
Klebsiella pneumonia	6
Proteus	5
Enterobacter	4

Conclusion

Moringa oleifera provides huge nutrition including proteins, carbohydrates and principle essential macrominerals supplements which are beneficial for the treatment and prevention of many human being diseases. There is need to introduce such types of medicinal plants, which may play huge role in new medicinal drugs in reducing hunger, malnutrition and poverty from malnourished areas of Pakistan.

Conflict of Interest. The authors declare no conflict of interest.

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The Morphological Characters of Egg and Relationship of Immature Stages of *Hermolaus modestus* (*Heteroptera*: *Pentatomidae*: *Eysarcorini*)

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(received November 1, 2019; revised February 24, 2020; accepted February 26, 2020)

Abstract. The paper investigates the biology and structure of egg and different developmental stages from 1st instar to 5th instar of *Hermolaus modestus* (Distant) (Pentatomidae: Eysarcorini). The experiment was conducted in the laboratory and the species was recognized as plant-sucking pest and mostly recorded on *Ocimum basilicum* (L.) from different areas of Karachi, Pakistan. The male and female individuals were breed under controlled laboratory condition. The maximum fertility range of *Hermolaus modestus* was observed 10 to 14 eggs per female and eggs were laid singly on the ventral side of plant leaves near the midrib. The incubation period was completed in 4 to 5 days. Newly emerged nymphs were dark red in colour but later colour changed into brown. The nymph passed through five instars progressively to complete the nymphal period. The first instar completed in 2 to 3 days with its specific characters. The second instar took 6 to 7 days with large clypeus. The third instar having scent gland plates and wing pads, completed in 5 to 6 days. The fourth and fifth instars of *H. modestus* completed in 5 to 6 and 8 to 9 days with prominent mesonotal and metanotal wing pads, respectively. The total nymphal period varied from 26 to 31 days. The total life cycle showed variation from 30 to 36 days. The total body length of male *Hermolaus modestus* was recorded as 4.8 mm to 5.0 mm.

Keywords: *Hermolaus modestus*, morphology of egg, pentatomidae, bugs, *Ocimum basilicum*

Introduction

Hermolaus modestus (Distant) belongs to superfamily Pentatomoidea, family Pentatomidae and genus *Hermolaus* (Kment and Carapezza, 2017). The Pentatomoidea is the large superfamily of Hemiptera, consisting of 1301 genera and 7182 species and 16 families in all over the world. Family Pentatomidae is the bug family and five antennal segments are the major character of this family. Pentatomids also famous as "stink bugs" which is invasive in nature. The family Pentatomidae consist of 4112 species (Schaefer and Panizzi, 2000), but recently Pentatomidae have 896 genera and 4722 species (Hassan *et al*., 2016). The Pentatomids also described as "shield bugs" due to their body is generally protected by a hard scutellum which covers half of the abdomen (Biswas *et al*., 2014). The *Hermolaus modestus* is closely similar to *Hermolaus ocimumi* and have some common characters like *Eysarcoris inconspicuous* (Heteroptera: Pentatomidae), found in the hills, plains, coastal and sub coastal areas of Pakistan. One of the encroaching member of this group, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) is very invasive species and widely observed in Japan, Korea Taiwan, eastern China and was also collected from the USA in 20th century, from Pennsylvania and Allentown in 2001 (Zhu *et al*., 2012). The isolated population of *Halyomorpha halys* (Stål) was collected from the west coast of the USA (Leskey *et al*., 2012a). *Halyomorpha halys* (Stål) was recorded on many host plants in all over the world especially in the US and China, they demolished the huge variety of economically valuable agricultural crops (Lee, 2015; Leskey *et al*., 2012b). Pentatomids are mostly phytophagous and severe pests of main crops. *Halyomorpha halys* was studied as polyphagous and herbivore in nature and found on diverse plants, including tree fruits, field crops and vegetables (Bergmann *et al*., 2016) and some species are predator and may be considered beneficial. However, *Hermolaus modestus* mostly recorded from grasses, fodder crops and vegetables.

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Hermolaus modestus is vulnerable sucking pests of fodder crops with piercing and sucking mouthparts. The adult and instars of *Hermolaus modestus* mostly recorded on *Ocimum basilicum* and main agricultural crops, like *Solanum melongena* (brinjal) *Hibiscus esculantus* (Okra Okra) and damage them (Hussain and Zahid, 2016). *Hermolaus modestus* badly damage the flowers and soft leaves of *Ocimum basilicum* plant by using their piercing and sucking mouthparts. Due to the piercing and sucking habit of pest, the growth of the plant is reduced. It is observed that most of the farmer grown *Ocimum basilicum* plant around the field of vegetables in Karachi, Pakistan. This practice is helpful to farmers for the protection of their main field crops from the attack of *Hermolaus modestus* and other vulnerable pests. Silva *et al*. (2014) studied the nymphal stages of *Euschistus heros* (Fabr.) (Hemiptera: Pentatomidae) and treated them with biological control agent *Telenomus podisi* under controlled environmental conditions. The biological comparison of *Chinavia impicticornis* and *C. ubica* (Hemiptera: Pentatomidae) pests and showed the fecundity table (Silva *et al*., 2015). The duration of development from egg to adult was calculated approximately 30 days for both species. The development of *Hermolaus modestus* was observed 30 to 32 days. The purpose of this study is to mention the similarities and differences of structure and developmental characters of *Hermolaus modestus*.

The *Ocimum basilicum* is commonly used for essential oil production. Hussain *et al*. (2008) studied the essential oil of *Ocimum basilicum* and found linalool component in abundant, which has antimicrobial and antioxidant properties. *Ocimum basilicum* has medicinal properties for heart asthma and blood diseases. This plant is antipyretic, carminative and expectorant in nature (Eftekhar *et al*., 2018). *Ocimum basilicum* is one of the most important limiting agent fodder crop for our livestock production. This enhancement research evaluates the fodder pest information for the betterment of good production of our livestock. The achievement of this study is to find out the new comprehensive information about invasive fodder pest. The knowledge of such information helps to develop the best understanding in the research field.

Materials and Methods

Site and climatology. The collection of *Hermolaus modestus* was performed in the early morning on different days from Karachi, Pakistan. For the purpose of insect collection, Faisal Cantonment and Gadap, Gulshan-e-Iqbal, Landhi, and Malir towns of Karachi were visited. It is observed that the population of *Hermolaus modestus* depends upon different environmental factors such as temperature, relative humidity and rainfall. Two major environmental factors temperature and relative humidity play a very important role in the successive development of immature stages of *H. modestus*.

Host plant. *Hermolaus modestus* (Distant) mostly collected from the plant, *Ocimum basilicum* (L.) and this species sometimes found on (Lucerne) *Medicago sativa* (L.) from different areas of Karachi Pakistan. *Ocimum basilicum* is one of the important fodder crops among genus *Ocimum* and mostly recorded from Indo-Pakistan. *Ocimum basilicum* is commonly called sweet basil and belong to family Lamiaceae. *Ocimum basilicum* has medicinal and economic importance.

Sampling. The beating method was used for the collection of *Hermolaus modestus* (adult). Beating method is the very significant and effective method for the collection of bugs and beetles (Metspalu *et al*., 2015) and generally adopted for the different insects' collection. Searching and picking method was applied for the collection of copulated pairs, eggs and instars from *Ocimum basilicum* plant. This technique was used by McCravy (2018). The study of *Hermolaus modestus* was performed in the laboratory at the Department of Zoology, Federal Urdu University Karachi. Temperature and relative humidity ranged from 20 to 32 °C and 35 to 75% respectively during the laboratory experiment.

Test management. The category of eggs, instars and copulating pairs of *Hermolaus modestus* was arranged in the laboratory after collection. Three washed Petri-dishes of 9 cm were arranged for experiment and 8 to 10 eggs and instars kept separately in each Petri-dish. The 10 pairs of adults arranged in each 12" × 16" sized chimneys. Chimneys washed and covered with a dull white clean muslin cloth. The leaves of *Ocimum basilicum* supplied as food adults and instars in alternative days. The temperature and relative humidity maintained from 20 to 32 °C and 35 to 75% respectively by a table lamp, small-sized motor fan, and wet cotton balls. Binocular microscope (Nikon SMZ-1) and cannon camera (A4000 IS) were used for the observation and taking photographs of adults, eggs and different larval stages of *Hermolaus modestus*.

Results and Discussion

Mating behaviour. Sometimes a male and female *Hermolaus modestus* were observed in the copulated condition in the laboratory. Male take initiates for copulation and reaches to the female. Both insects move and touch their antennae in very slow motion. The male turns around to the female and contact in the end to end position and then insert its pygophore in the female genital hole. It was observed that female remains motionless during all process. The eggs were laid 10 to 14 in numbers per female.

Egg. The egg of *H. modestus* was observed milky white when freshly laid and then gradually changed into dark red. Sixteen bright micropyles and horizontal lines observed in mature egg. The length and width of the egg were measured 0.6 mm and 0.4 mm respectively (Fig. 1). The varied incubation period of *H. modestus* was observed from 4 to 5 days. The newly hatched instars were collected and shifted carefully by using a wet camel hairbrush on host plant fresh leaves in the Petri dishes.

First instar. The first instar lasted for 2 to 3 days. The body of the first instar impunctate and oval in shape; pronotum dark brown; abdomen light brown; clypeus generally longer than paraclypeus; labium passing the third coxae; antennae four segmented, 1stand 2nd segments of antenna being fused, thick, pinkish-red (Fig. 2).

Second instar. This stage almost the same in shape with 1st instar; colour comparatively lighter than 1st instar. This stage was completed in 6 to 7 days. The mesonotal wing pad was starting to develop; clypeus long and deflexed; antenna dark brown (Fig. 3).

Third instar. The third instar was also occupied 5 to 6 days. Abdominal turga of the third stage was lighter in colour than thoracic turga; scent gland plates dark brown; wing pads extending to cover at least first abdominal segment; antennal segment having a ratio of 1=2=3=4<5 (Fig. 4).

Fourth instar. The fourth stage of larvae completed in 5 to 6 days. Body colour lighter than previous; meso and metanotal wing pads almost equal in length and reached to third abdominal segment; the ratio of antennal segment size in the order of 1= 2 < 3 = 4 < 5 (Fig. 5).

Fifth instar. The fifth larval period consisted of 8 to 9 days. The meso and metanotal wing pads clearly observed in this stage. Mesonotal wing pad longer than metanotal wing pad and antennal segments ratio was 1 = 2 < 3 < 4 < 5. The legs were ochraceous and hairy (Fig. 6).

Fig. 1. Egg of H. modestus.

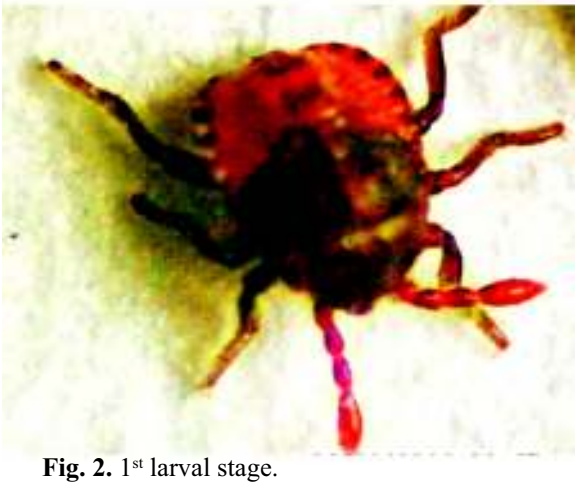

Fig. 2. 1st larval stage.

Fig. 3. 2nd larval stage.

Adult. Body, dark brown punctate and ochraceous; head length (1.07 mm) slightly smaller than length of pronotum (1.12 mm), pronotum width 2.5 mm; scutellum long as 1.6 mm and 1.8 mm in width; antennal formula is 1< 2 = 3 < 4 < 5. The range of length in adult males was 4.8 to 5.0 mm. (Fig. 7, Table 1).

Key to the various immature stages of *Hermolaus modestus*:

1. Mesonotal wing pad present in the initial stage; clypeus almost deflexed and long; antenna dark brown 2
   Wing pads developed and reaching to first abdominal segment; scent gland plates developed 3
2. Colour lighter than the previous stage; mesonotal wing pad developed to appear ..... 2nd stage.
   Pronotum dark brown than abdomen and body almost oval in shape; 1st and 2nd antennal segment fused ..... 1st stage.
3. Thoracic turga dark in colour; scent gland plates dark brown; all antennal segments equal in size except 5th segment, which is slightly larger than 4th segment ..... 3rd stage.
   Body-colour lighter than previous stages; meso and metanotal wing pads different in size and clearly observed; 1st and 2nd antennal segments equal in size 4
4. Abdominal and thoracic turga almost light in colour; mesonotal and metanotal wing pads equal in size and reached to 3rd abdominal segment; 1st, 2nd antennal segments, and 3rd, 4th antennal segments equal in size ..... 4th stage.
   Mesonotal wing pad larger than metanotal wing pad; 1st and 2nd antennal segments same in size; 3rd, 4th and 5th antennal segments larger in sequence ..... 5th stage.

Table 1. Measurements of the body parts of adult *Hermolaus modestus* (♂)

Body parts	Male (♂) (mm)
Body length (BL)	4.8
Head length (HL)	1.07
Head width across eyes (HW)	1.9
Length of 1st antennomere (A1)	0.23
Length of 2nd antennomere (A2)	0.3
Length of 3rd antennomere (A3)	0.3
Length of 4th antennomere (A4)	0.46
Length of 5th antennomere (A5)	0.63
Pronotum length (PL)	1.12
Pronotum width (PW)	2.5
Scutellum length (SL)	1.6
Scutellum width (SW)	1.8
Abdomen of length (ABL)	1.2
Abdomen of width (ABW)	2.02

Fig. 4. 3rd larval stage.

Fig. 5. 4th larval stage.

Fig. 6. 5th larval stage.

The structure of egg and developmental stages of *Hermolaus modestus* was the same as in other species of Eysarcorini. Ali and Rizvi (2010) determined the development of *Coocinella septempunctata* at specific temperature and humidity. Aziz *et al*. (2013) studied

Fig. 7. *Hermolaus modestus* (adult).

the life cycle of *Trilocha virescence* and estimated 33 days of development. *Hermolaus modestus* was showed variation and completed the life cycle in 30 to 32 days, under controlled laboratory conditions. The mortalities were gradually decreased from early to late stages during development. The temperature and relative humidity were observed as important factors which influenced the egg hatching time and developmental activities of *H. modestus*. Haye *et al*. (2014) studied the phenology and life table of *Halyomorpha halys*. The populations of *H. halys* evolved within 33.2, 42.3 and 75.8 days from egg to adult under controlled temperatures of 30, 25 and 20 °C, respectively. Many researchers described the importance of temperature and relative humidity for developmental activities in different species and agreed that the effect of temperature speed-up the development process in species. Rastogi and Pandey (2008) observed the effect of different constant temperature on the life-history of *Zygogramma bicolorata*. Michels and Flanders (1992) described the larval development of aphid and observed the importance of temperature influence. The *H. modestus* showed comparatively variation than *Eysarcoris inconspicuus* (H. Sch.) (Heteroptera: Pentatominae). The egg length and width in *Eysarcoris inconspicuus* were 0.9 mm and 0.5 mm respectively and micropyles were invisible in *Eysarcoris inconspicuus* (Koppel *et al*., 2009). The egg of *H. modestus* was dark red in colour with 16 visible micropyles and barreled in shape. The egg size of *Hermolaus modestus* was observed 0.6 mm and 0.4 mm in length and width respectively. The operculum and sixteen visible bright micropyles distinctly observed on the anterior side in the egg of *Hermolaus modestus*. The length of paraclypeus was small than clypeus with the prominent labium reaching to third coxae in the first instar of *H. modestus*. In the second instar, the development of mesonotal wing pad was almost going to start and clypeus prominently large in size. In later stages, scent gland plates were developed and wing pads reached to the first abdominal segment. The sizes of mesonotal and metanotal wing pads were the same in length. The width of scutellum slightly short than broad in adult *H. modestus*. This character was different as compared to *Hermolaus capitatus* (Distant). The length of head comparatively less than the length of pronotum in *H. modestus* and showed the similarity with *Hermolaus ishurdiensis* (sp. nov). The 2nd antennal segment was larger than 1st antennal segment in *H. modestus*. The 3rd antennal segment was equal to 2nd antennal segment and 4th antennal segment was larger than the 3rd antennal segment. The 5th antennal segment was larger than 4th in the adult of *H. modestus*. The total length of the antenna in the adult male of *H. modestus* was almost 1.92 mm.

Conclusion

The biological characters of *Hermolaus modestus* showed somewhat variation with the other member of this group. The female laid eggs singly near the midrib of plant leaf in 10 to 14 numbers. The emergence of the first instar in 4 to 5 days. The first instar occupied 2 to 3 days having small paraclypeus than clypeus and large labium toward the third coxae. The second instar developed in 6 to 7 days with prominent large clypeus. The third instar having scent gland plates and wing pads, accomplished in 5 to 6 days. The fourth instar of *H. modestus* completed in 5 to 6 days with the character of equal size mesonotal and metanotal wing pads. The fifth instar took 8 to 9 days with long mesonotal wing pad. The total nymphal developmental period showed variation from 26 to 31 days. The total life cycle of *Hermolaus modestus* was completed in 30 to 36 days. The body of an adult male was dark brown and punctate with 1.07 mm, 1.12 mm and 1.6 mm of head, pronotum and scutellum length respectively. The total body length of an adult male was 4.8 to 5.0 mm.

Conflict of interest. The authors declare no conflict of interest.

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Pak. j. sci. ind. res. Ser. B: biol. sci. 2021 **64B**(1) 13-17

Efficient Protocol for *In vitro* Regeneration of *Ocimum sanctum* using Nodal Segments as Explants

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(received January 26, 2018; revised December 12, 2018; accepted December 13, 2018)

Abstract. *Ocimum sanctum* commonly called (holy basil) an herb containing medicinal, ornamental values, is often used in culinary applications. This research focuses on the improved and efficient protocol for the direct regeneration and acclimatisation of *Ocimum sanctum* using nodal segments. Organogenesis and multiplication from explants were observed to a maximum on Murashige and Skoog (MS) medium supplemented with 0.1 mg/L of 6-Benzyl amino purine (BAP) and 0.025 mg/L of Indole-3-acetic acid (IAA). Furthermore, same medium was found effective for the induction of roots, in the *in-vitro* grown plantlets. A series of experiments were conducted to optimise the acclimatisation of *in-vitro* grown rooted plantlets of *Ocimum sanctum.* For this study different types of potting mix in assorted ratios were used to obtain best supporting media for the acclimatisation, A7 media containing soil : farmyard manure (75:25) and A1 media containing (100%) sand were found best supporting medium for the acclimatisation and hardening of *Ocimum sanctum.*

Keywords: organogenesis, nodal segments, acclimatization, regeneration, *Ocimum sanctum*

Introduction

Aromatic plants have played a significant role in the combating diseases, since ancient times, including, *O. sanctum* is a great contender for new investigations due to vast array of activities (Triveni *et al.*, 2013). *Ocimum sanctum* L. (holy basil) is considered a very sacred plant and rich source of essential oil (Kumar *et al*., 2011). The chief component is eugenol and the oil contains other chemical compounds like l- methyl chavicol, cineole, citral, 1- 8-cineole, carvacrol, ∝-pinene, eugenol, eugenol methyl ether, methyl eugenol, *p*-cymene, linalool, bornyl acetate, and eugenol (Kothari *et al*., 2004). Essential oils and herbal extracts have great potential as natural flavours and enormous uses in traditional practices. Ursolic acid is the principal component of the *O. sanctum* (tulsi) leaves (Shanmugam *et al*., 2013; Fontanay *et al*., 2008). *O. sanctum* used in various purposes such as leaves, flowers, stem, root, seeds etc. are known to have potential pharmacological activity. It also plays a significant role in treatment of fevers, arthritis, convulsions, bronchitis etc. in traditional medical practices (Kumar *et al*., 2011).

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Therapeutically, *O. sanctum* is used for treatment of eye diseases and its oil also helps in eye sight improvement (Rajeswari, 1992), while its extract have been shown to possess antimalarial, insectical and larvicidal activity (Kumar *et al*., 2011). It also has anthelmintic (Sen, 1993), antidiabetic (Hannan *et al*., 2015), analgesic, antioxidant (Khanna and Bhatia, 2003; Rajeswari, 1992), immune-modulatory (Jeba *et al*., 2011), antiulcer (Dharmani *et al*., 2004), hepatoprotective (Chattopadhyay *et al*., 1992) and anti-inflammatory activity (Malick, 2014).

There are previous reports available on *O. sanctum in-vitro* propagation using other explants sources, but this is the first report of *in-vitro* multiplication using nodal segments as explants. The main rationale of this study was to develop a sterile germ-plasm source that can be used for other *in-vitro* studies like bio-transformation (Zafar *et al*., 2012) and protoplast manipulations.

Material and Methods

Plant material. Seeds were collected from the *O. sanctum* plants grown in the green house of International Center for Chemical and Biological Sciences (ICCBS), University of Karachi.

Sterilization. Seeds of *O. sanctum* were surface sterilized using 20% (v/v) commercial bleach in autoclaved distilled water for 20 min with continuous shaking. Two drops of Tween-20 were also added with the sterilant as a surfactant. The seeds were then sieved through a pre-sterilized tea strainer and rinsed thrice with autoclaved distilled water in a petri-dish (Khan *et al*., 2008). Finally, seeds were placed on a filter paper in a petri-dish to remove moisture. The entire experiment was performed in a laminar flow cabinet.

Seed germination. For germination, MS medium (Murashige and Skoog, 1962) was used with 25 g/L sugar and 6 g/L agar without plant growth regulators (PGRs). The pH of the media was adjusted to 5.75 before autoclaving. Media sterilization was performed by autoclaving at standard temperature and pressure (121°C, 15 p.s.i., and 15 min). The seeds were transferred to the germination media and the cultures were kept under 16:8 photoperiod provided by white fluorescent light (1000 Lux) at 25±1 °C for 4 weeks.

Shoot induction and multiplication. Nodal segments (1-2 cm) of *Ocimum sanctum* were taken from the seed germination medium under aseptic condition. 28 days old nodal segments were cultured on shooting and multiplication medium.

The media were corresponding with the formulation of MS medium containing BAP and Kinetin (KN) in various concentration having media code OS1 – OS8 as shown in Table 1. MS medium was considered as control without growth regulators. All the cultures were incubated at 25±1 °C with 16:8 photo periods. Data of the plant growth parameters *i.e.*, shoot regeneration; average number and length of shoot were recorded weekly for five consistent weeks.

Root induction. For *In vitro* root propagation, *Ocimum sanctum* shoots were taken on various roots induction media in order to propose an optimized medium for root induction shown in (Table 2) The media was formulated with MS media containing various kinds of auxins IAA, 1-naphthelene acetic acid (NAA) and indole-3-butyric acid (IBA) individually, and in combination with varied concentration (Table 2). In this experimental setup, Ten different combinations bearing media code OR1 to OR9 were used, while basal MS medium with no growth regulators was control.

Auxin-cytokinin combination media. To test the effect of auxin:cytokinin combination medium on the plant growth, *viz-a-viz* shooting and rooting, an experimental set up shown in Table 3 was applied under controlled environment for five weeks.

Table 1. Effect of cytokinins on shoot induction in *O. sanctum*

Code	Cytokinin (mg/L)		% Shoot regeneration/ explant	No. of shoots*	Average shoot length (cm)*
	BAP	KN			
MS	0.0	0.0	54	2.73±0.34	1.80±0.24
OS1	0.05	0.0	87	9.98±0.09	4.93±0.15
OS2	**0.1**	0.0	**100**	**11.06±0.22**	**5.53±0.13**
OS3	0.25	0.0	100	11.00±0.23	5.23±0.12
OS4	0.5	0.0	100	10.86±0.24	4.96±0.15
OS5	1	0.0	67	10.46±0.23	4.61±0.24
OS6	0.0	0.25	67	4.03±0.03	2.30±0.18
OS7	0.0	0.5	75	4.83±0.14	3.03±0.19
OS8	0.0	1	87	5.03±0.07	3.96±0.07

*Values are mean ± standard error (X ± SE)

Table 2. Effect of auxins on root induction

Code	Auxins (mg/L)			% Root induction/ explant	No. of roots*	Average root length (cm)*
	IAA	IBA	NAA			
MS	0.0	0.0	0.0	60	2.06±0.33	2.00±0.27
OR1	0.1	0.0	0.0	100	4.00±0.23	4.86±0.09
OR2	0.5	0.0	0.0	100	4.60±0.23	4.40±0.13
OR3	1	0.0	0.0	100	5.80±0.10	3.76±0.20
OR4	0.0	0.1	0.0	93.3	9.93±0.18	4.87±0.08
OR5	0.0	0.5	0.0	100	11.06±0.15	5.01±0.05
OR6	0.0	**1**	0.0	**100**	**12.93±0.22**	**5.96±0.03**
OR7	0.0	0.0	0.1	93.3	8.60±0.23	4.73±0.11
OR8	0.0	0.0	0.5	100	9.20±0.14	5.06±0.12
OR9	0.0	0.0	1	100	12.06±0.18	5.93±0.20

*Values are mean ± standard error (X ± SE)

Acclimatization. Acclimatization of *Ocimum sanctum* plantlets was done in which different supporting materials *i.e.* soil, charcoal, farm yard manure (FYM) were used in different ratios and combinations as shown in Table 4.

Statistical analysis. All the parameters of recorded data for each treatment were analyzed by analysis of variance (ANOVA) in MSTAT-C software (MSTATC, 1989). Fifteen replicates were used for each treatment applied.

Results and Discussion

In this study, a modified protocol of direct regeneration and acclimatisation of *Ocimum sanctum* plantlets was

Table 3. Effect of synergistic/single medium on direct regeneration in *O. sanctum*

Code	BAP	IAA	No. of shoots	Average shoot length (cm)*	No. of roots*	Average root length (cm)*
OC1	**0.1**	**0.025**	**12.40±0.22**	**5.94±0.05**	**15.00±0.23**	**6.05±0.06**
OC2	0.1	0.05	11.33±0.12	5.59±0.24	14.46±0.13	5.83±0.11
OC3	0.1	0.1	10.65±0.12	5.38±0.24	13.06±0.06	5.10±0.24
OC4	0.1	0.25	10.10±0.22	5.02±0.24	12.13±0.09	4.98±0.12
OC5	0.1	0.5	10.03±0.26	4.83±0.11	10.93±0.06	4.55±0.12

*Values are mean ± standard error (X ± SE)

Table 4. Acclimatization of *Ocimum sanctum*

Code	Formulation	Ratio	% survival/leaf colour and morphology
A1	**Soil**	**100**	**87%, green/normal**
A2	Charcoal	100	5%, green/normal
A3	FYM	100	50%, green/normal
A4	Soil: Charcoal	75:25	70%, green/normal
A5	Soil: Charcoal	50:50	60%, light green/stunted
A6	Soil: Charcoal	25:75	50%, light green/stunted
A7	**Soil: FYM**	**75:25**	**87%, green/normal**
A8	Soil: FYM	50:50	75%, green/normal
A9	Soil: FYM	25:75	60%, green/normal
A10	FYM: Charcoal	75:25	60%, light green/normal
A11	FYM: Charcoal	50:50	50%, light green/stunted
A12	FYM: Charcoal	25:75	35%, light green/stunted

optimized. The germination and development of seeds were recorded in MS medium after three weeks of culture. The auxiliary bud explants showed 100% regeneration after 21 days of culture when placed in media supplemented with auxins and cytokinins alone and in combination.

Shoot induction and multiplication. In this study, the cultured nodal segments on MS medium with cytokinins (BAP and KN) showed budding. Concentration and cytokinin type both exhibited their role in successful *in-vitro* growth. There are some reports of favouring KN over BAP previously (Kumar and Rao, 2007; Bhattacharya and Bhattacharya, 2001), however, Begum *et al*. (2000) and Gopi *et al*. (2006) reported direct multiple shoots differentiation of *O. sanctum* and *O. gratissimum* L. respectively in BAP supplemented medium. Preece (1995) stated that apical dominance and cell division due to cytokinin, influences the growth and shoot induction. The maximum induction of multiple shoots after four weeks of initiation (11.06±0.22) was attained on medium (OS_2) containing 0.1 mg/L BAP, having 5.53±0.13 cm average shoot length. As the concentration got higher, the corresponding number of shoot and shoot lengths were on gradual decrement, while application of KN did not yield any remarkable results (Table 1).

Root induction. After four weeks, the regenerated shoots attained the height (up to 1-1.5 cm). Root induction occurs when regenerated shoots were transferred on rooting media which contains MS media with different concentration of auxins (IBA, IAA, NAA) as shown in Table 2. The comparative study of different auxins shows that the root induction response is vigorous in IBA as compared to IAA and NAA. The optimum media type was thus determined. A relationship was found between the rooting percentage and number of roots *in-vitro* differentiated plant was found in Table 2. The results showed that, the 100% root formation with increase in number and length was observed in OR6 medium which contains IBA 1 mg/L i.e., 12.93+0.22 root induction and average root length is 5.96±0.03 cm as shown in Table 2. Concurrently, it is also observed that the effect of IAA and NAA during the induction of roots gives 100% root induction with undersized root number and length. Deklerk *et al.* (1997) suggested that high concentration inhibits, while lower auxin concentrations having growth stimulating effect on root induction.

Auxin. *Cytokinin medium effect on plant regeneration*. In third set of experiment, auxin in combination with optimized cytokinin i.e., BAP 0.1 mg/L (OC2) was evaluated to testify synergistic medium potential (Table 3). Emergence of shoot buds was observed on MS medium having combination of BAP and IAA illustrated the good response (Fig. 1a). It is observed that analysis of variance revealed highly significant differences in OS1 medium formulated with (IAA 0.025 mg/L and BAP 0.1 mg/L) respectively, which showed vigorous effect on shoot multiplication (12.40±0.22) and shoot length (5.94±0.05 cm) as observed (Fig. 1b and c). Auxin (IAA) oxidized and metabolized swiftly, supporting the formation of shoots and embryos hence low concentration of IAA encourage root growth and retain the apical dominance (George, 1993). Cytokinins contribute a vital role in plant regulation which provokes calli division in the presence of auxin, leading to bud or root formation directly on the explants or from calli (Taiz and Zeiger, 2004). The synergistic effect of auxins and cytokinins has also been reported in other *Ocimum* spp. (Phippen and Simon, 2000; Singh and Sehgal 1999;

Patnaik and Chand, 1996; Vasil and Thorpe, 1994; Evans *et al*., 1981). In the same medium, rooting was vigorous when the shoots were placed in medium supplemented with IAA and BAP in combination. The number of roots induced was 15.00±0.23 having average root length 6.05±0.06 cm (Table 3). New individual plantlets propagated well from axillary buds on further multiplication (Fig. 1d).

Acclimatization. Regenerated shoots having vigorous roots from rooting media were transferred to green house for acclimatization. Three different types of potting mix (soil, charcoal and farm yard manure) were used in different ratio as shown in Table 4. In order to study the phenotypic variation of *in-vitro* propagated plantlets in *ex-vitro* conditions. In this experimental design, it is observed that the A7 media containing Soil: FYM at the ratio of 75:25 showed 87% survival rate and plantlets were healthy and green with normal proliferation, whereas A1 media containing 100% sand also exhibited optimum growth for the acclimatization and hardening of *Ocimum sanctum,* albeit the foliage colour was on the lighter green shade. Previous reports showed that acclimated plants survival under *ex vitro* condition ranges from 70% (Begum *et al*., 2000) to 85% (Singh and Sehgal, 1999), but as the waxy cuticle layer of young plants develop, they become more resistant to environmental stresses (Khan *et al*., 2007).

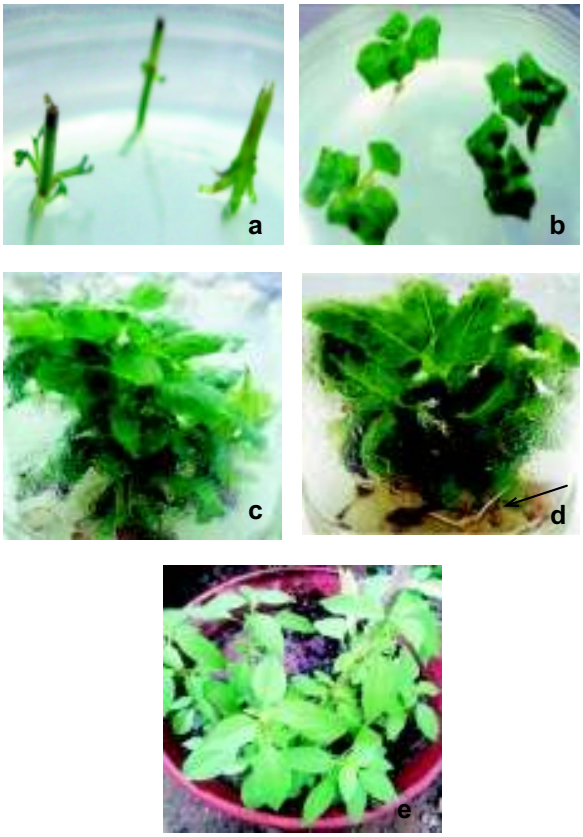

Fig. 1. Direct regeneration in *Ocimum sanctum* (a) budding of nodal segments after one week (b) plantlet formation after three weeks (c) shoot multiplication (d) rooting (arrow) and plantlet formation (e) acclimatized plant.

Conclusion

In this study, the identical plantlets of *O. sanctum* were produced in massive quantity from nodal segment which reveal that direct regeneration is more efficient than indirect (callus) regeneration method. Shoots were vigorously propagated from nodal segment under *in-vitro* conditions, which lead to root proliferation on optimized MS medium supplemented with Growth regulators (BAP and IAA) respectively. This study is concluded with the approach for producing the identical plantlets of *O. sanctum* in immense number from nodal segments.

Conflict of Interest. The authors declare no conflict of interest.

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Pak. j. sci. ind. res. Ser. B: biol. sci. 2021 **64B**(1) 18-24

The Development of Mucosal Immunization as an Alternative Approach for Production of Antisera Against Saw Scale Viper from Sindh

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(received August 22, 2019; revised November 24, 2019; accepted November 27, 2019)

Abstract. The reported cases of snake bite are 5.4 million per year, making the serious envenoming issue so, that WHO categorises cases of snake bite in neglected tropical diseases. Anti snake venom is produced by intramuscular injection of crude venom with different formulation of adjuvants to equine animals, but these intramuscular injections have several serious health impact in equine. Snake species categorised according to geographical locations, and there is a different composition of the snake venom at different locations. So, this research had been designed and evaluated new oral adjuvant formulation for viper family of Sindh, Pakistan. This is a animal immune based analysis consisting two Saw scale viper from Thar and costal area of Sindh because both have different habitat and morphological appearance. The venom toxin protein, combined with a mineral oil adjuvant for oral immunization. The adjuvant with venom *via* oral route produced of specific I_gG and horse give maximum response with three doses of venom. The antibody titer was measured by ELISA showed significant results in both groups. For confirmation of specific antibodies isolated from horse serum, serum neutalization assay and immunodiffusion test were carried out. The snake venom with oral adjuvant immunization, is safe, efficient and time saving for production of equine Immunoglobulins I_gG.

Keywords: oral immunization, venoms, saw-scale viper, immunoglobulins (I_gG)

Introduction

The snake bite cases from Thar parker district of Pakistan are very distinct from other snake bite cases because of difference in snake variety. The saw-scaled vipers are the primary causes of snakebite morbidity and mortality in the desert area of Sindh, Pakistan. Similar circumtances are reported from African, Middle east countries, which creating problem in handling of snake bite cases Pouyani *et al*. (2016).

The worldwide estimated snake bite cases are 5.4 million out of which 2.5 million envenomation and 125,000 deaths per year. It is estimated that there are more than 1,000,000 snake bites in India alone leading to between 45000 to 50000 deaths per year Menno *et al*. (2017). South Asia has the highest rate of snake bite cases. India has highest mortality index; Pakistan has no accurate available data, but approximately 40,000 reported cases of snakebites and 8000 fatal cases per year are recorded by a literature search. In SriLanka, approximately 33,000 cases per annum, and in Nepal 1,000 death per year recorded. The fatality rate is more or less same in the Asian region that is about 20% Parveen *et al*. (2017)

This serious envenoming problem and increase numbers of deaths has led WHO to recognise it as one of the neglected tropical diseases, chiefly accepted as an occupational hazard and a disease of poverty. So, the investigator started to design and achieve a better consideration of the compositional difference of venoms and assist the production of affordable, effective antivenoms against the majority of venomous snakes to solve the problem. The most effective treatment for snake envenomation is the administration of particular antivenom scince many decades, which remains scarce in many parts of the world. Ratanabanangkoon *et al*. (2016)

Until launching a pharmaceutical product with complete identification, purity, protection and efficacy profiles anti-snake venoms development must follow the requirements of Good Manufacturing Practices (GMPs), including animal ethics. The identity of anti-snake venoms is determined by the equine animal species used as a source of immunoglobulin and the snake venoms source used as immunogens, León *et al*. (2018)

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Worldwide, many immunization methods have been discovered, such as recombinant toxins, synthetic peptides, or just the isolalion of antigenic epitopes by different molecular digestion techniques, but in case of snake venom, the venomous animals essential to keep in captivity antivenom production open the era of new immunization approaches is considered relatively possible and adapted by manufacturers without introducing significant changes. Furthermore, since antivenoms gained by using novel immunization strategies have no differences as obtain from traditional antivenoms. The regulating bodies' endorsement processes should not restrain their product in the market. However, with these benefits, there is batch-to-batch difference is still expected, as a importance of immune response of immunized animals, Bermúdez *et al*. (2018)

In Pakistan, Echis carinatus (saw-scaled viper) are commonly found in desert areas, especially in agriculture land, calltle shades (Ther, Cholistan, and Makran in Baluchistan). It is a flat body with short tapered tail measuring about 0.4 to.6 meters long that travels rapidly in the opposite direction of the air flow. Echis carinatus have different variations of colours such as gray, olive or brown tone, back and some times both sides have different colours, with small white spots, (Parveen *et al*., 2017; Odedara, 2017).

Saw-scale viper venom act as potent procoagulant toxins, which lead to venom-induced consumption coagulopathy (VICC) is the significant clinical manifestations in humans. Venom activates the clotting pathway by procoagulant toxins, resulting in clotting factor defect. The procoagulant toxin type varies between snakes species and can activate factor X and V, prothrombin activator or consume fibrinogen. (Rogalski *et al*., 2017; Berling and Isbiter, 2015).

Therefore, the present study designed to establish a new strategy oral immunization in order to enhance the titer of antibodies against the venom of two saw-scale viper found in Sindh, Pakistan. As adjuvants are used for many decades for enhancement of immunogen activity, and in animals these adjuvants are used for production of hyperimmune sera, Valverde *et al*. (2017). Oil based vaccine are commonly practiced for veterinary controllable diseases which is designed for single dose for life long immune response against diseases studied by Park *et al*. (2016) The researchers have been started working out the oral immunization technique, especially for the cure of *Mycobacterium tuberculosis*. So, we also design the mucosal immunization technique for production of specific anti snake venom against local viper family of Sindh, (Chambers *et al*., 2017; Beltrán-Beck *et al*., 2014).

Materials and Methods

Ethical considerations. All experimental horses were kept in the departmental of laboratory animal sciences Ojha campus, Dow University of Health sciences under the supervision of equine veterinarians. Ethical approval letter No (IRB-685/DUHS approval/2016/184) for the present study has been taken from Institutional Board Review Committee, Dow University of Health Sciences, Karachi.

Venom collection. Saw-scale viper have different groups which also have a different morphology and habitat. We collected venomous saw scale vipers from Ketti Bander, Kachall Border, Jhang, Qader Loo, Jatti, Shah Latife, Chill Mehar, (coastal areas) and Therparker district of Sindh (Fig. 1-2). The costal area viper are still not categories as species or sub species of saw-scale viper its need another detail study but these snake have their on habitat. All snakes were kept in the snake house at the Laboratory Animal Science Department, DUHS.

Venom was collected mennually with standard protocol provided by WHO. Then venom was centrifuged at 2500 RPM for 30 min. for cleaning the sample, freeze dried and stored at – 20 °C till used.

Fig. 1. Saw-scale viper of costal area (Sindh).

Fig. 2. Saw-scale viper of Tharparker (Sindh).

Animal study plan. In breed line NIMRI mice strain weighting from 20–30 g were selected for venom dose calculation (lethality testing and neutralization Assays), and Local horses age ranges from 3 to 8 years, weight 200–300 Kg was included in this study for immunization protocol, purchase from local farmers after certification by a veterinarian.

All experimental groups (large and small animals) were kept in Laboratory Animal Science Department, DUHS under supervision of veterinary consultant.

Venom protein estimation. The venom protein was estimated by standard Bradford method. The basic principle of this assay is the conversion of Coomassie Brilliant Blue G-250 dye into different colours that is neutral (green), anionic (blue) and cationic (red). The existence of protein in the dye forms a strong, non-covalent complex with the protein's carboxyl group by Van der Waals forces and amino group through electrostatic interactions, which is measured by the use of a calorimetric absorbance reading. Cheng *et al*. (2016) (Bradford kit provided by Thermo Scientific^{TM})

- Tharparker saw-scale viper protein calculated as 90µg/µL of venom.
- Coastal saw-scale viper protein calculated as 120 µg/µL of venom.

The venom protein was calculated for LD^{100} in mice. Hosseini *et al*. (2017)

Toxicological studies. Toxicological study was done in two groups of NIMRI mice. Each dilution tested on five mice with one control. One group for coastal saw-scale viper and 2nd group for Tharparker district saw-scale viper (SSV) which are shown in Table 1.

The venom was serially diluted by 1:9 ratio and from each dilution injected to five mice by intraperitoneal route. Dead or alive mice were observed and recorded from 24 to 48 h, respectively. WHO guidelines (2010).

Animal husbandry conditions. Good animal handeling, high nutritional diet, water and biological stress free conditions were provided. Daily veterinary examination and treatment have been provided as per requirement.

Preparations of adjuvants. The small animals NIMRI mice was used to find out the ideal concentration of (adjuvants with venom protein combinations) to develop an oral adjuvant formulation by (v/v) Tween^{20} Tween^{80}, Mineral oil, PBS and saw-scale snake venom. These were mixed with a vortex mixture till dense creamy white or light brown colour (emulsion) appeared, Shah *et al*. (2015). The orally by 0.5 mL from each ratio given feeding tube in five mice for tolaratability, shown in Table 2.

Protocol for monovalent immunization of horses. Ten horses were allotted for research trial as given a unique number, name and after passes the quarantine time, horses distributed in two groups each group, have five in for protocol 1 and protocol No. 2 (Thar SSV and costal area SSV respectively). Prior to immunization of horses, serum was collected from all animals and stored as negative controls, shown in Fig. 1.

Isolation and testing of horse I_gG. We use caprylic acid for precipitation technique for commercial production of anti-snake venom. Quantification of horse

Table 1. Toxicological studies into two groups.

Snake venom	Dilution 1 0.5mL/mice	Dilution 2 0.5mL/mice	Dilution 3 0.5mL/mice	Route of injection 0.5mL/mice	Control
Costal SSV	0.5	0.5	0.5	Intraperitoneal	0.1
Thar SSV	0.5	0.5	0.5	Intraperitoneal	0.1

Table 2. Preparation of venom protein formulation

Snake Venom fixed volume	Adjuvant ratio1 10% (v/v) 0.5 mL/mice	Adjuvant ratio 2 20% (v/v) 0.5 mL/mice	Adjuvant ratio 3 30% (v/v) 0.5 mL/mice	Route of injection	Control
Costal SSV	05	05	05	Orally	01
Thar SSV	05	05	05	Orally	01

I_gG against snake venoms in the different groups of horses sera was analyzed by modifying enzyme link immuno sorbent assay (ELISA), the absorbance values read at 492 nm with the help of ELISA reader, and the results were recorded.

The specific immunoglobulin against snake venoms, neutralised by agar gel immunodiffusion test was performed using an Ouchterlony double immunodiffusion technique which is shown in Fig. 3.

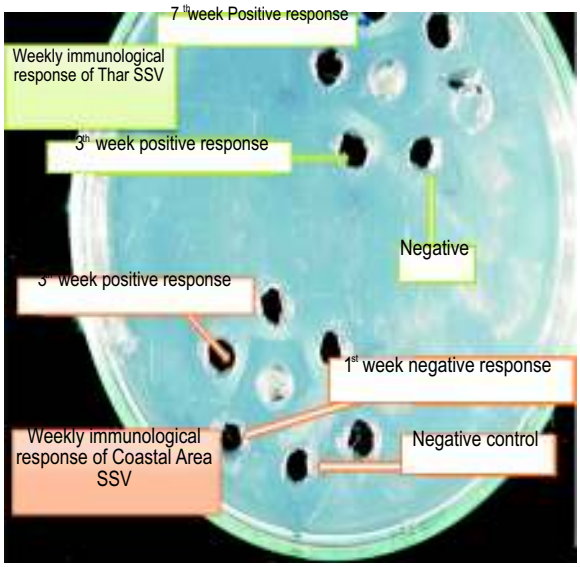

Fig. 3. Immunodiffusion results of Thar and coastal area saw-scale viper.

Statistical analysis. All data were calculated and analysed by SPSS version 16 with paired sample T test for standard deviation.

Results and Discussion

LD^{100} for snake venom. In mouse strains, the mortality of saw-scale viper snake venom was checked. It was found that intraperitoneal route was ideal in NIMRI mice for calculation of LD^{100}. The result of LD^{100} of costal area SSV venom was 480 µg /25 g body weight of mice and for Thar SSV venom was 360 µg/25 g.

Table 4 and Fig. 4 shows the antibodies (IgG) titer measured by ELISA. The result was statistically

Table 4. Comparison of antivenom of Thar SSV with costal area SSV produced by oral adjuvant measured by ELISA

Weeks	Antivenom of Thar SSV (µg/µL of IgG)	Antivenom of costal area SSV (µg/µL of IgG)
0 week (Pre-venom) bleed	0.0	0.0
1 week after the first dose	0.6	0.8
3 weeks after Second dose	1.7	1.9
7 weeks after 3rd dose	2.9	3.1

*Elisa (absorbance at 492 nm); Statistically significant differences between oral adjuvant ($P < 0.058$).

Table 3. Protocol for monovalent immunization of Horses

Immunization week	Adjuvant composition	Protocol 1 Thar SSV (venom)	Protocol 2 costal area SSV (venom)	Route
1	Tween20 & Tween80 Mineral oil, PBS	500 µL	500 µL	Orally
3	Tween20 & Tween80 Mineral oils, PBS	250 µL	250 µL	Orally
7	Tween20 & Tween80 Mineral oils, PBS	200 µL	200 µL	Orally

Table 5. Serum neutralization assay of Thar SSV venom by *in vivo* studies

Weeks	Fixed amount of LD^{100} venom (µL)	I_gG Antibodies (µL)	Vivo studies	Live mice (Mice)	Dead mice	Control mice one for each group	Remarks
0 Week (Prevenom)	40	150	5	----	5	Alive	
After 1st week	40	150	5	----	5	Alive	
3rd Week	40	150	5	4	1	Alive	80%
7th Week	40	150	5	5	0	Alive	100%

Table 6. Serum neutralization assay of costal area SSV venom by *in vivo* studies

Weeks	Fixed amount of LD^{100} venom (µL)	I_gG Antibodies (µL)	Vivo studies (Mice)	Live mice	Dead mice	Control mice one for each group	Remarks
0 Week (Prevenom)	40	150	5	----	5	Alive	
After 1st week	40	150	5	----	5	Alive	
3rd Week	40	150	5	4	1	Alive	80%
7th Week	40	150	5	5	0	Alive	100%

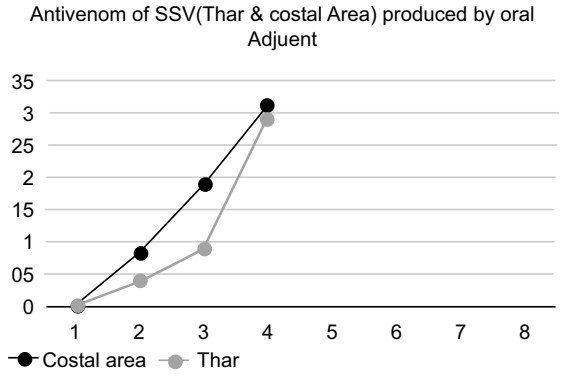

Fig. 4. Comparison of antivenom of Thar SSV with costal area SSV produced by oral adjuvant measured by ELISA.

significant (P valve 0.058) means both the groups of SSV responds very well with mucosal immunization technique given by oral route.

Table 5 and 6 showed serum neutralization assay *in vivo* studies. Both groups of SSV venom produced sufficient antibodies when gave as oral adjuvant formulation and provided 100% protection after three doses of immunization.

Conclusion

There are no reference articles worldwide for use of anti-snake venom preparation, so the present study was carried out to develop an oral adjuvant formulation for delivery of snake venom to mucosal surface which have well defined lymphoid system for production of Anti snake venom immunoglobulin which also have the same potential as by the injectable path.

The majority of licensed vaccines was administered designed for subcutaneous or intramuscular injection for activation of systemic humoral immunity (*e.g*. antibody production) against specific antigen, with partial cellular immunity (*e.g*. T cell-mediated) and at mucosal surfaces only produce poor protection. Studied by Ramirez *et al*. (2017)

In contrast, in oral immunization the pathogen or toxin direct interact with mucosal surfaces induces successfully mucosal antibodies (I_gA) and cell-mediated immune responses which ultimately activate systemic antibody response (I_gG), Newsted *et al*. (2015)

Since, many decade several adjuvants have been used for several decades to develop stronger immune response with least adverse effects. These adjuvants induce better response to immune system than other formulation. Our findings strongly support the efficacy of oral adjuvant-carrier system for the effective induction of a specific immune response and targeted delivery system for production of anti-venom immunoglobulin described by Mehrabi *et al*. (2018)

More than 70 years adjuvants are use to design vaccines to improve the immune response to particular antigens,

increase the duration of antibody response, and decrease the frequency of vaccination doses and the amount of antigen in each dose. Although water-in-oil emulsions are used for the long-term production of humoral immune response in poultry vaccines, as they are not suitable to be used in human vaccines, because they induce chronic inflammatory responce at the injection site stu died by Jafari *et al*. (2017) So, we used oil emulsion for oral route vaccination in equine animals Meenatchisun-daram *et al*. (2008) showed in his research the maximum titer of immunoglobulin I_gG against saw-scale viper concentration varied in the ranged of 0.3–6.7 mg/mL of I_gG for Russell's viper venom, and 0.5–6.9 mg/mL of I_gG for Saw-scaled viper venom throughout the immunization period. This study is comparable with our study which shows Immunoglobulin G titer is 0.6 -2.9 ug/uL for Thar saw-scale viper and 0.8- 3.1ug/uL for costal area saw-scale viper.

Funding statement. This study was financially funded by Sindh Govt Health Department ADP scheme number 33 on going sero-biology, project establishment and research approval letter IRB-685/DUHS/ Approval/ 2016/184 from Dow University of Health Sciences.

Conflict of Interest. The authors declare no conflict of interest.

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Pak. j. sci. ind. res. Ser. B: biol. sci. 2021 **64B**(1) 25-29

Physico-chemical Analysis, Total Polyphenolic Content and Antioxidant Capacity of Yellow Dye Extracted from *Curcuma longa*

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(received February 23, 2019; revised February 9, 2020; accepted March 27, 2020)

Abstract. Turmeric (*Curcuma longa*) is a well known condiment of the Asian cuisine and also used as ayurvedic medicine in the content, since ancient times due to its potential therapeutic properties. Main colouring constituent of *Curcuma longa* is curcumin and curcuminoids. In the present work natural yellow dye was extracted from rhizome of turmeric using an effective low cost method of solvent extraction. The developed natural yellow dye was assessed for physico-chemical analysis, toxicity, polyphenolic content and antioxidant activity. Physico-chemical assay showed good nutritional profile of the extracted natural yellow dye and quite safe at dose level 3.5g/Kg body wt. Significant phenolic content was found to be 63.32 mg GAE/100g, and also showed potent antioxidant capacity (% inhibition) ranging from 5.1-20.4 at 1-5 mg/mL concentration. Animal trials showed no mortality in the mice.

Keywords: *Curcuma longa*, curcumin, toxicity, phenolic contents, antioxidant activity

Introduction

Turmeric (*Curcumin longa* L), a member of the ginger family of herbs (*Zingiberaceae*), is a widely used spice that is native to the south of Asia. Most of the curcuma species grow in mountainous areas of the World, but some common species are often cultivated in gardens and used as a spice, food preservative and colouring, flavouring agent to the food and as medicinal plants (Zdrojewicz *et al*., 2017; Vyas, 2015). Curcumin is obtained from the dried rhizome of the plant *Curcuma longa*, first isolated almost two centuries ago and its structure was determined in 1910. Curcumin is the active ingredient of turmeric, which is used daily in Indian and other south Asian cuisines as a spice. Most commercial turmeric preparations consist of ~2-8% active curcumin (Choudhary and Sekhon, 2012). The main compounds in turmeric include curcumin (1E, 6E)-1, 7-Bis (4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3, 5-dione) and two curcuminoids, de-methoxycurcumin and bis-demethoxy curcumin (Fig. 1).

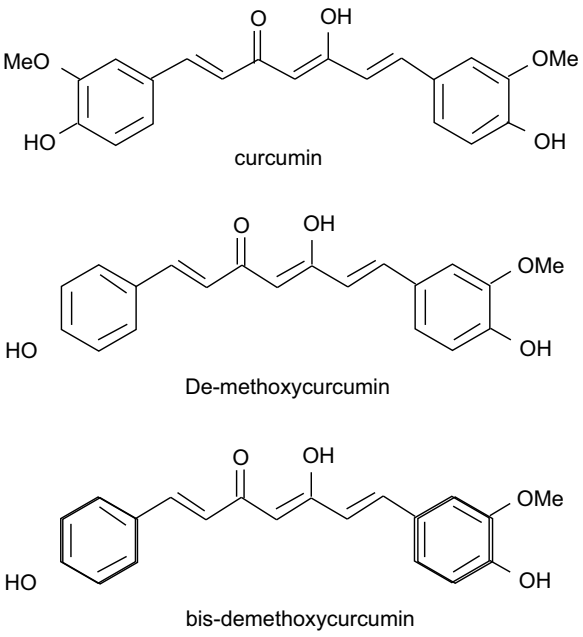

Fig. 1. Structures of curcumin and curcuminoids.

Curcumin and curcuminoids contribute the yellow colour to turmeric and have received increasing attention because of their many bio-activities. Current research shows that curcumin and curcuminoids have antifungal, anti-bacterial, anti-inflammatory, antioxidant, anti-mutagenicity and cholesterol lowering activities (Akter *et al*., 2019; Amalraj *et al*., 2017; and , 2017; *et al*., 2017). They can prevent rheumatoid arthritis in animal model (Funk *et al*., 2006). Oral administration of 5 and 10 mg/Kg curcumin significantly reduced the duration of immobility in depressive-like behaviours (tail suspension and forced swimming) in mice (Xu *et al*., 2005). Pre-treatment with curcumin significantly

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enhanced the rate of wound contraction, decreased mean wound healing time, increased synthesis of collagen, hexosamines, DNA and nitric oxide, and improved fibroblast and vascular densities (Jagetia *et al*., 2004).

Antioxidants are used in food industry to inhibit or delay the oxidation. Antioxidant found in plants are based upon constituent nutrients with demonstrated radical-scavenging capacities as well as upon non-vitamin or mineral substances. Plant based medicines contain flavonoids, polyphenols and flavoproteins which act as potent antioxidants like alpha-tocopherol, ascorbate and carotenoids (Kamble and Gacche, 2019). Further, some plants or specific combinations of herbs in formulations may act as antioxidants by exerting super-oxide scavenging activity or by increasing superoxide dismutase activity in various tissue sites Sawant *et al*., 2009). These groups of compounds are substances that may exert cell-protective action by more than one biochemical mechanism. In addition to antioxidant properties, cancer-protective factors are found in many plants including some fruits, vegetables and herbs (Alok *et al*., 2014).

Curcumin is main compound in turmeric and it act as free radical scavenger as well as hydrogen donor, binds with metals particularly iron and copper (Typek *et al*., 2019; Hatchera *et al*., 2012). Curcumin effectively inhibits intracellular amyloid toxicity at low dosages in rats due to its free radical scavenging activity (Ye and Zhang 2012). However, it is also effective in various models of antioxidant such as DPPH scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, ferric ions reducing power and ferrous ions chelating Ak and Gulcin (2008). The objectives of this research were physico-chemical analysis and to determine the total phenolic content (TPC) by using Folin-Ciocalteau method and antioxidant capacity of yellow dye extracted from turmeric by DPPH-free radical scavenging method.

Materials and Methods

Plant materials. Fresh plant turmeric (*Curcuma longa*) was purchased from local market of Lahore, Pakistan. Folin-Ciocalteu's (FC) phenol reagent was obtained from Merck (Darmstadt, Germany). Sodium carbonate, gallic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma (Steinheim, Germany).

Pigment extraction: Solvent extraction of curcumin was carried out using water. Solid liquid extraction was carried out by blending the sliced material with water using rhizome, solvent ratio of 1:5 (by mass per volume). The juice was then filtered to remove particulates. The filtrate was again used for further blending of the material. Slight acidification of the extraction medium done by addition of 0.15% ascorbic acid, 0.1% citric acid enhances curcumin stability. The filtrate was dried in hot air oven at 30-35 °C to form a dry product. The dry product was then ground using a rotating blade grinder to form a powder product that was passed through a 60 mesh sieve. After drying the residual weight was noted down and the amount of curcumin being extracted is calculated and then analyzed.

Physico-chemical analysis. Physico-chemical analysis like moisture, ash, fat fibre, protein contents were measured by AOAC methods (2016).

Animal studies /toxicological studies. Albino male mices weighing 25-30 g with ages of 2-3 month were used in this study and were fed with standard diet and water. They were kept in clean and dry cages and maintained in well ventilated animal house with 12 h light-12 h dark cycle. The animals were randomized into control and experimental groups and divided into 4 groups each group of 5 mice. Animals in group 1 were treated with distilled water. Animals in groups 2, 3 & 4 were treated with curcumin water extract 0.5, 1.5 and 3.5 g/Kg body wt.

Determination of total phenolic contents. Total phenolic contents of curcumin extracts were determined using Folin-Ciocalteu reagent as described by Singlaton and Rossi (1965). Turmeric extract (0.1 mL) were mixed thoroughly with 0.5 mL Folin-Ciocalteu reagent after 5 mins, 1.4 mL of 7.5% sodium carbonate (Na_2CO_3) was added and allowed to react for 90 min at room temperature. The absorbance was measured at 760 nm using spectrophotometer (UV-1700, Shimadzu Japan). Samples were measured in three replicates. Standard curve of gallic acid solution (10, 20, 40, 60, 80 and 100 ppm) was prepared using the similar procedure. The results were expressed as mg GAE/100 g extract sample.

DPPH assay. The antioxidant activities of curcumin extracts were evaluated through free radical scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The determination was based on the method proposed by Akowuah *et al*. (2005). Three mL of 0.004% DPPH methanolic solution was added into 100 µL of sample extracts. The mixture was thoroughly mixed and kept in the dark for 30 min. The control was prepared using

3 mL of DPPH. The absorbance was measured at 517 nm using spectrophotometer (UV-1700, Shimadzu Japan). Samples were measured in three replicates. Percentage of DPPH scavenging activity was calculated as:

% inhibition of DPPH = [Abs control –(Abs sample/Abs control) × 100.

Statistical analysis. Experiment data were analyzed using Excel (Microsoft Inc.) and SPSS version 17.0 software. Significant differences between samples were analyzed using analysis of variance (ANOVA). Data obtained were reported as mean ± standard deviation (Steel *et al*., 1997).

Results and Discission

Unquestionably, the colour is a vital constituent of food. It is one of the rapid and indispensable factors depicting the quality and acceptability by the consumer. Generally artificial colours are added to improve the appearance of processed and preserved food products. Normally it has been appreciated that where natural colour of food is unattractive colouring matter may be added. In the present study natural yellow colour was extracted and assed for physico-chemical analysis, polyphenolic contents, toxicity and antioxidant activity.

Physico-chemical analysis of natural yellow dye. Table 1 shows the nutritional composition of the natural yellow dye. The basic nutritional compositions i.e. moisture, ash, protein, fat and total fiber were analyzed. The moisture and ash contents were found to be 11.76% and 1.83% respectively. Protein contents were 12.98% in yellow curcumin dye. 2.03% fat and 0.87% fiber contents were found in prepared yellow dye. The obtained nutritional analysis results suggest that the dye extracted from turmeric can be considered a potential dye to be used as natural colourant.

Table 1. Nutritional composition of the natural yellow dye

Parameters	Value (%)
Moisture	11.76 ± 0.8
Ash	1.83 ± 0.2
Protein	12.98 ± 0.9
Fat	2.03 ± 0.3
Fibre	0.87 ± 0.1

Data are presented ± SD

Toxicological studies. Toxicological studies were conducted on healthy mice to check the harmful effects of dye prepared. The oral dose of dye in pure drinking water was given in different quantities to animals and it was found that after 4 week the mice remained alive at dose 3.5 g/Kg body wt and also their weights were increased normally. It was found that there was no mortality and it is quite safe at said dose level.

Polyphenolic contents. Total phenolic contents of plants extract were tested using the diluted Folin-Ciocalteu reagent. Result clearly showed that curcumin had the total phenolic content with mean value of 63.32 mg GAE/100 g extract. Several studies (Wong *et al*., 2006; Wu *et al*., 2006; Shan *et al*., 2005) reported that phenolic compounds in spices and herbs significantly contributed to their antioxidant properties.

Antioxidant capacity. The antioxidant capacity of curcumin is attributed to its unique conjugated structure, which exists in an equilibrium between the diketo and keto-enol forms that are strongly favuored by intramolecular H-bonding Weber *et al*. (2005). Since de-methoxycurcumin and bis-demethoxycurcumin have similar structures like curcumin. The antioxidant capacity in term of % inhibition (DPPH) were range from 5.1-20.4 at 1-5 mg/mL concentration (Fig. 2). Curcumin shows typical radical-trapping ability as a chain-breaking antioxidant. Generally, the non-enzymatic antioxidant process of the phenolic material form the non-radical product (Tangkanakul *et al*., 2009; Chattopadhyay *et al*., 2004). Further studied by Masuda *et al*. (2001), the antioxidant mechanism of curcumin using linoleate as an oxidizable poly unsaturated lipid and proposed that the mechanism involved oxidative coupling reaction

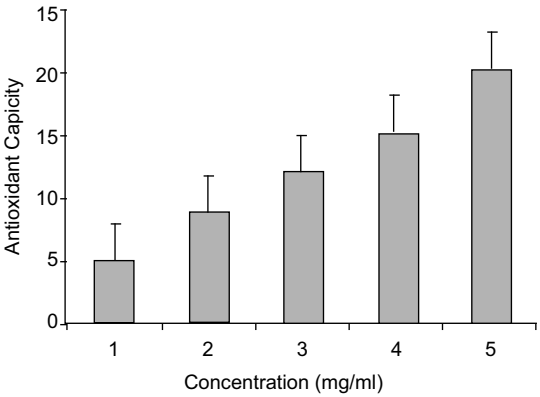

Fig. 2. Antioxidant capacity (% Inhibition) of curcumin.

at the 3 position of the curcumin with the lipid and a subsequent intramolecular Diels-Alder reaction.

Conclusion

In conclusion, it was found that the natural yellow dye (curcumin) was non toxic, nutritive and have potent free radical scavenging activity.

Acknowledgment

The authors would like to thanks the Food and Biotechnology Research Center (FBRC), PCSIR Labs Complex, Lahore, Pakistan for providing all the facilities during this period of work.

Conflict of Interest. The authors declare no conflict of interest.

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Solar Dryer for Large/Medium Scale Ripening and Drying of Dates; A Case Study of Dates Growing Area of Pakistan

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(received July 13, 2017; revised January 22, 2019; accepted January 24, 2019)

Abstract. This study describe the design features and performance of the unglazed transpired solar dryer for large and medium scale drying of Dates in the Dates growing area of Pakistan. The dryer has the designed capacity to dry 500 Kg of Dates from Khalaal (70% humidity) to Tamar (25% humidity), at 55 °C ± 5 °C in 72 continuous hours using biomass furnace as an auxiliary heat source and it covers approximately 256 m^2 areas. This is the ideal humidity condition to store Dates for one year without any further degradation. In traditional open sunshine the huge quantity of Dates are dried in over 150 h hours and requires hectare of area to spread the Dates. Moreover, the quality of Dates in the open sunshine is affected badly due to two main reasons. Firstly, the heavy dust in the area that sticks the Dates during the drying process makes them unable to eat. Secondly, the Monsoon rains in the months of July and August also destroys Dates placed in the open areas for drying. Dera Ismail (D.I.) Khan, a remote district of Khyber Pakhtoon Khawa province of Pakistan, was selected to study the performance of Dates dryer, where solar insolation is 700-750 W/m^2 in the months of July and August. The Dates were dried continuously and during off sunshine hours biomass auxiliary source was used. This solar dryer, containing eight trolleys and each trolley is loaded with twelve trays, is the first in Pakistan to dry a large amount of Dates. Moreover, the unglazed transpired dryer is designed in such a way that it can withstand the dusty atmosphere of D.I. Khan and also protect the Dates from dust and rainy water during the drying period. The design does not have any adverse effect on the quality of dried Dates. In D.I. Khan around 350 days are sunny per year and solar thermal technology is economically feasible compared to other conventional energy resources with a payback period of 3 and 7.5 years compared to un-subsided and subsided cost of Natural Gas respectively. The Natural Gas is the cheapest conventional energy source in Pakistan. But solar thermal technology is yet not popular source of energy in the area due to lack of information, fear of initial capital cost and weak government policies for renewable energies. This study also incorporates the recommendations to overcome these issues regarding Solar Thermal Technology, PACS Number: 44.40.+a, 89.30.Cc, 84.60.-h, 89.30.-g.

Keywords: thermal radiation, solar power, direct energy conversion, energy resources

Introduction

Agriculture forms are the base of Pakistan's economy, which produces large quantities of grains, fruits and vegetables. Around 70% of the 177 million population of Pakistan is employed in agriculture sector and it is the major source of income and foreign exchange for the country Qazi (2011). Pakistan is blessed with a tropical climate in Dates growing areas, ideal for production of Dates, and it is fourth in the world in Dates production. Here the annual production of Dates is about 535,000 tones which are partially exported and mostly it is locally consumed. There are more than 300 varieties of Dates in Pakistan. Out of these large number of varieties, Dhakki of Dera Ismail Khan, Begam Jangi of Balochistan and Aseel of Sindh are the varieties which have high international demand due to their exotic taste. Lack of post harvesting processing facilities make these valuable varieties which are highly demand in European and Asian countries, unable to get its worth in the export market (The Express Tribune Pakistan, 2011).

Pakistan, being a developing country, mainly depends on traditional harvesting and post harvesting techniques. As a result lower quality of agriculture products are produced and formers have to face economic losses. The modernization of post harvesting techniques using sustainable and low cost solar technologies offers

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opportunities to stimulate economic growth and this is aim of this study. But due to the inadequate post harvest care, it is estimated that about 20-30 percent of the produce is wasted and the remaining product is of lower quality for human consumption. The traditionally harvested Dates contain dust and insect infection and their quality is so poor that it does not meet the criteria of International market to export and earn foreign exchange. Due to the substandard quality, the Dates farmers are unable to get reasonable price in the local market as a result of living standard of the poor people in remote areas is affected. If solar dryers are used to remove excess moisture from the Dates before storage, then their quality will be enhanced and insect infestation will be minimized. Similarly, large quantities of excess Dates, now being wasted could be solar dried in a controlled environment and would be available to export and for use during off-season. Solar dryer technology is well developed and can be used for bulk drying in a controlled environment. Solar dryers involve a nominal initial cost but they produce better looking, better tasting, and more nutritious foods Boubekri *et al*. (2009). The food value of Dates and their marketability is enhanced. The dryers make the drying process much faster, safer and efficient than traditional sun drying techniques.

In Pakistan Dates processing by solar dryer is under exploited. Perishable farm produce are wasted or sold at throw-away prices during the peak seasons due to inadequate post harvest storage facilities and lack of effective processing or preservation techniques. On the other side marketing problems for Dates in Pakistan is same as it is in other Date producing countries. These include irregular production, poor choice of cultivars, crop damage caused by insect pests and plant pathogens, poor crop quality due to traditional harvesting and drying methods, lack of post harvest technology, insufficient organization of marketing process, and intensive market competition of imported Dates Nejjar (1999). The ripening season for Dates starts with the rise in summer temperature in June and August is the peak production period. Unfortunately the Monsoon and dust stormy seasons also falls within these months of the year. This is real bottleneck for this crop to harvest and store. This solar dryer has been designed to address the above mentioned environmental issues and dry Dates at required temperature and humidity.

The traditional methods are still popular for ripening/ curing the fruits and vegetables for obvious reasons of high cost and unavailability of conventional energy and lack information about emerging solar drying technologies. The Dates at the Dong stage are spread on mats and exposed to sun in open air and time for complete ripening and drying of the Dates in the Pakistan is very long due to dusty atmosphere. Normally it takes more than one and half week to dry Dates in the open sun as at night the temperature of the Dates drops and it takes time to reach optimal temperature of drying next day provided there is no rain in that period. The quality of traditionally sun-dried product under dusty condition becomes very poor and non-uniform with low yield. Due to persistent rain and stormy condition a large amount of the harvested Dates become moldy, fermented, and dusty and damaged by bird and insects. The growers become disheartened and consequently the rate of further cultivation and propagation of Dates product is being impaired badly. Due to the waste of crop, people have trend to make Chuharas (cut dried Dates) and export to neighbouring countries like India at throw away prices to save post harvest loses in the production of Tamar (complete) Dates.

The conventional energy sources like electricity, natural gas and hydrocarbons are in acute shortage of supply in Pakistan and very expensive to be used for drying fruits and vegetables. Today, Pakistan is facing more than 5000 MW electricity shortfall and about 900 MMcfd shortfalls in the Natural Gas (WAPDA, 2011) data. Both the shortfalls are overcome through load management plans on daily basis. On the other side, the cost of conventional energy in Pakistan like electricity and natural gas is unaffordable for farmers to dry Dates. Further more, farmers are socially reluctant to adopt new harvesting technologies like solar drying due the fear of higher technological cost. Fortunately, Pakistan is blessed with plenty of sunshine in Dates and vegetable growing areas. The solar intensity in the under study area varies from 700 W/ m^2 to 750 W/m^2 for about 8 hours per day in the Dates growing season and the same have been confirmed in this study as well Sukhera *et al*. (1986). This energy is abundant, free of cost and requires simple technology to dry Dates. For this study solar energy and biomass, both are abundant and freely available in the area, have been used to continuously dry Dates in shortest possible time by using minimum space to easily handle bulk amount of Dates. This solar dryer design is very simple to copy and fabricate using locally available material and semiskilled manpower.

In this study the attempts to understand the socio-economic importance of Dates ripening and drying in Pakistan to formulate appropriate interventions to improve the sector. This is being done by using freely available solar energy, biomass energy, simple dryer design with optimum performance and socially acceptable to adopt and reproduce these dryers. The main beneficiaries will include Dates producers who will focus on their production to meet the national and international market demand of better quality dried Dates. Processors/farmers will have better access to technologies, processing techniques and to market demand information. Researchers and other service providers will be able to focus on factors that are of economic importance to the fruit-drying sector, issued rose during the drying process and latest developments in solar thermal technologies.

A large medium size solar dryer for average farmers in Pakistan has been designed, fabricated and tested for drying of Dates keeping in view the simplicity of fabrication and use with optimal results. The design allows the use of minimum space for Dates to spread and uses locally available resources like sun and biomass to dry Dates in continuous short interval. Solar energy as a clean and cheep energy source is proved to be an alternatives of conventional energy especially in sun-belt countries like Pakistan for solar drying. This typical dryer uses solar and biomass energy for continuous heating and electricity for exhaust fans to remove humidity from the chambers. The results are very encouraging and this design of solar dryer is being tested in other part of the country to ensure its suitability against dust, rain and minor variation in solar intensity for all parts of the Dates growing areas of Pakistan.

Material and Methods

Drying techniques and methodology. This type of dryers use solar and biomass energy to heat the air in the drying chamber to flows over the Dates. The relative humidity of the heated air in the chamber decreases and is capable to hold more moisture form the Dates. This moisture is captured from the Dates by the dry air and the same is vented through exhaust chamber. This process continues till the desired amount of moisture is maintained in the Dates. It is worth to mention that the required temperature must be maintained for drying Dates failing to which the quality of dry Dates would be affected. In this particular case 55 °C±5 °C chamber temperature is essential for drying from Rutab to Tamar. For the ease of users, the temperature and humidity of other fruits and vegetables has also been mentioned in the Table. 1 [**http://www.fao.org/docrep/t0681E/t0681e05.htm**].

With the passage of time, the actual amount of moisture evaporated per unit of time decreases. Initially, the moisture from the outer surfaces of the Dates evapourates till dried. The moisture from the inner surface of the Dates has to move to the top surface and it takes time which slows the drying process. During the drying of inner surface, overheating on the upper surface may occur due to the slower rate of moisture evaporation. If the temperature is more than 50 °C±5 °C in this period, the Dates quality would be badly affected. To avoid overheating during this inner surface drying of the Dates, the rate of airflows through exhaust fan was controlled to maintain required temperature. However, Khalal stage Dates is very temperature sensitive and to ripe Khalal Dates to Rutab by heating, the chamber temperature was maintained to 55 °C±5 °C for 10 h in a day and no biomass auxiliary was used at night to avoid overheating and this was repeated for two consecutive days and data has been recorded for sunny period of 20 h. After the careful removal of moisture from the most outer surface, the continuous drying started using auxiliary biomass till complete drying.

For this study, the most abundant Dates growing area like D. I. Khan was selected to investigate the performance of the dryers in real conditions. The

Table 1. Maximum permissible temperature for drying agricultural produce and their initial and final moisture contents.

Produce	Moisture content (%)		Max. permissible temperature (°C)
	Initial	Final	
Dates	70	25	50-55
Chilies	80	5	65
Onions	80	4	55
Potatoes	75	13	75
Apple	80	24	70
Apricots	85	18	65
Green peas	80	5	65
Green beans	70	5	75
Cauliflower	80	6	65
Cabbage	80	4	55
Tomatoes	96	10	60
Brinjal	95	6	60
Peaches	85	18	65
Grapes	80	15-20	70

Table 2. Dates developmental stages & their characteristics

Stages	Detail of Dates	Required Temperature for drying & Humidity	Picture
Khalaal	Yellowish colour, 19-25 weeks Moisture 60%	T: 50-55°C For ripening of Dates	
Rutab	Golden brown, Sugar partially inverted, juicy and soft tissue, 26-28 week, Moisture 35%	T: 50-55°C For drying of Dates	
Tamar	Dark Brown Complete Dates 29 week Moisture 23-25%	T: 50-55°C Complete Dried Dates	

temperature and humidity required to dry the Dates is given in the Table. 2 and this paper describe the study of Khalal Dates drying to Tamar Dates. Thermo-hygrometers were installed in two chambers to monitor the temperature and humidity at one hour interval. The ambient temperature and humidity were also recorded at the same interval. The intensity of the light was recorded with the help of illuminometer and it remained 700-750 W/m^2 in the months of July and August but it has not been presented in hourly data in the paper.

The community participation has been ensured and local farmers were trained to take over the dryer after completion of the study. The feedbacks form the farmers were also taken and addressed in the paper. Therefore, the social and geographical issues have been discussed in this paper along with recommendations to make solar drying technology popular in Dates growing areas.

Dryer design. Active solar dryers using exhaust fan to move the hot air from collector to the drying chamber has been used. In the developing countries flat plat solar energy collector are used in dryers for its lesser complication in design and operation. These kind of solar collectors with metal sheet unglazed absorbers had collector efficiencies up to 30% and reached temperature rise of only about 50 °C (at ambient 35-40 °C). While, glass cover absorbers have improved efficiencies to 60% and dryer temperature rises to 60 °C (at ambient 35-40 °C) studied by Jensen *et al*. (2001). However, further addition of the glazing and insulation just increases the cost of the collector for solar dryer.

The unglazed transpired collector (UTC) developed for this study comprises of commercial black-coated perforated plate. Ambient air is forced into the plenum through the holes in the plate as shown in Fig. 1. It is worth to mention that the UTC does not require any glazing and insulation while, reduced the cost of the collector significantly. Collector efficiency has been noted between 50 to 70 % at a temperature between 50 to 60 °C (at ambient 35-40 °C). Studies by Kutscher *et al*. have highlighted the performance of the UTC as a low-cost alternative to glazed flat plate solar collectors (Kutscher *et al*., 1993 and 1991). Limited investigation by Meer and Machlin concluded that the UTC would be an appropriate technology for drying applications Meer and Machlin (2001). Under the conditions, the UTC dryer seems to be appropriate and economically viable option to select for Dates dryers. Therefore, the design of this type of collectors is presented here along with the problems faced during the real study.

The specifications of the dryer are given below: Actual collector area = 60 square meters and (20 collector having 3 square-meters each); Perforation diameter = 2.0 mm; Plenum depth= 10 cm; Electrical power required = Six fans of 40 Watts each

The hybrid UTC dryer was designed with the biomass furnace as an auxiliary heat source. This ensures the continuous operation of the dryer during clouds season at the night. The hot air from the collector enter into the dryer chamber which passes through the trays and takes the moisture from the dates being dried. The

Fig. 1. Picture of the complete solar dryer.

exhaust vent located on top of the drying chamber expels the moisture in the air by the use of exhaust fans. The biomass furnace ignites the hot air through the primary air inlet during off sunshine period. Convective heat transfer from the fins and outer surface of the heat exchanger to the air entering the drying chamber raises the effective temperature within the chamber. The heated air circulates through the drying chamber and evaporates the moisture from the Dates on the drying trays and exits through the exhaust vent as described earlier. The pipe type heat exchanger built-into the primary air inlet of the furnace heats the delivery system and combustion rate of the furnace is regulated by the amount of air flow into the furnace. The Fig. 1-3 show the pictures of the complete dryer, trays and Dates in Khalaal and Tamar stages respectively.

Fig. 2. Picture of the trays of the solar dryer (Khalaal stage).

Fig. 3. Picture of the dried (Tamar) Dates.

The dryer was design to dry 500 Kg of Dates in one run by reducing humidity from 70% to 25% The relevant equation to determine the mass of water lost from the Dates is (Gatea, 2011).

$$MW = M_C [(W_I - W_F)/(100 - W_F)]$$

where:

W_I = the initial moisture content; W_F = the final moisture content; M_C = the initial mass of the crop; M_W = the mass of the moisture evapourated and therefore is 500 [(70 - 25)/(100 - 25)] = 300 Kg. It is assumed that each Kg of water requires 2.5 MJ of heat for its removal, then the total heat needed is 300 x 2.5 = 750 MJ.

Results and Discussion

To prove that the design is appropriate and capable to dry the Dates at 50-60 °C within 72 h the ambient temperature and humidity were recorded along with humidity and temperatures of, Chamber-1 and Chamber-2. In this study we selected Khalaal (humidity 70%), Dates to dry them to Tamar shapes (humidity 25%). However, Dates can also be picked from the Dates plants in the shapes of Rutab shapes (humidity 35%) to dry them to Tamar.

Chamber temperature. The Dates were dried to Tamar in continuous 72 h and the first reading in the data was taken at 8.00 a.m. The data consist of the ambient temperature, temperatures of Chamber 1 and Chamber-2. The ambient temperature starts rising at 8.00 a.m. and reaches it's maximum at 38 °C at 2.00 pm and then decreases to 24 °C at 2.00 am. To supply uninterrupted heat to Dates, the auxiliary heat was supplied to the drier chambers after 6.00 pm using biomass energy. It can be seen from Fig. 4 that the temperature in both the chambers remained similar and constant at around 52-56 °C during the entire 72 h of drying process except few short interval Peaks. It shows that both the chambers are receiving same amount of energy and it is the reflection of successful designing. Three peaks in chambers temperature were observed (Fig. 4) due to the plenty of sunshine at 2.00 pm and rise of ambient temperature that resulted in lower thermal heat losses. However, these peaks are of short duration and did not exceed the upper control limit of chamber temperature that might adversely affect the drying quality of the Dates. However, this can be avoided by covering few of the solar collectors of the dryer but we have ignored

it for the sake of actual data this time. From Fig. 4 it is observed that the design of the dryer with exhaust fans is appropriate to keep the chamber temperature constant at 55 °C to 60 °C during the entire 72 h.

Chamber humidity. The ambient humidity of the chamber changes along with the ambient temperature in day and night cycle. The humidity is maximum at mid night (2.00 a.m.) and decreases to minimum at mid day time (2.00 p.m.). From Fig. 5, it can be seen that humidity of the chamber increases during initial 12 h due to the evapouration of water particles from the upper surface of the Dates in Khalal shape. However, after the saturated evaporation, the Dates starts drying and the humidity decrease constantly with the passage of time. After 45-50 h the humidity of Dates reaches to 35% and the Dates are in Rutab shapes and are ready for consumption as fresh Dates. It is worth to mention that in Pakistan Dates are conventionally picked in Rutab form as well from the Dates plants. Therefore, within 25 h these Dates can be completely dried to Tamar shapes. This design is effective, consistent and produces quality dried dated in specified minimum time.

Economic considerations. The major source of energy in Pakistan is Natural Gas which is locally produced and comparatively cheaper than other energy sources like electricity, oil or coal. It has major share of 44% in the energy mix of Pakistan (OGDCL Annual Report, 2012). However, it is not available in remote areas of the country for use. In these days Pakistan is facing

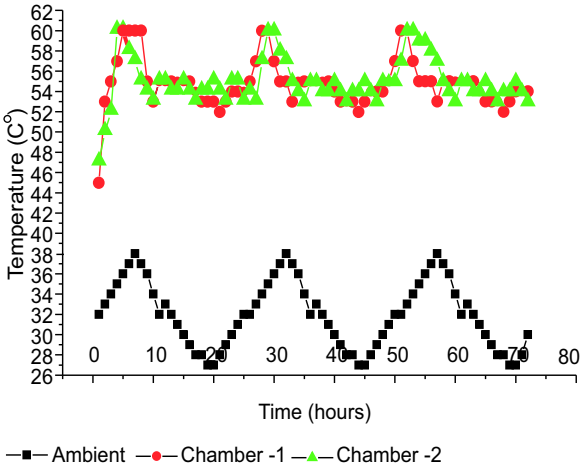

Fig. 4. Temperature profile of dryer chamber and ambient temperature.

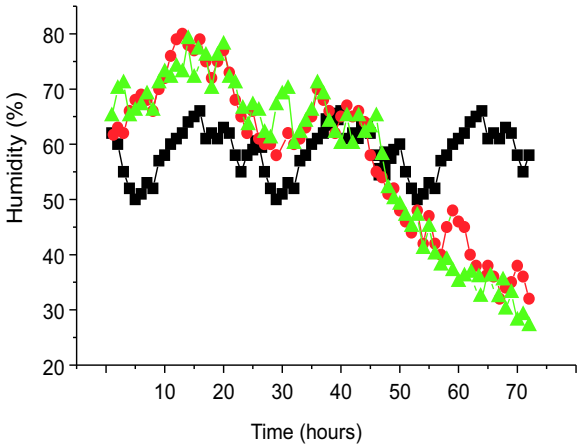

Fig. 5. Humidity profiles of dryer chamber and ambient humidity.

acute shortage of natural gas due to its lower cost and heavy consumption in electricity generation and industrial utilization like fertilizers. Natural gas being a cheapest energy source has been compared with solar dryer cost to verify that solar thermal energy is economically feasible in Pakistan. The natural gas heating value over here is 40MJ/m^3 with burning efficiency of 60%.

The perforated collectors have an average efficiency of 50% and average annual solar insolation in D. I. Khan Pakistan is 5.7 kWh/m^2d; and it is available for 350 days per year. Then from a surface area of 60 m^2 it should deliver 5.7 x 0.4 x 60 x 350 x 3.6 MJ = 172368 MJ.

The natural gas heating delivered from the Burner would be 172368/ (40 x 0.60) = 7182 per m^3
Current natural gas domestic subsided price in Pakistan; Rs = 190 per MMBTU
Cost of the gas is Rs = 32756
Current natural gas price in Pakistan; Rs = 463 per MMBTU
Cost of the gas is Rs = 79636
Cost of the dryer Rs = 250,000
Payback period compared with subsided gas 7.6 years
Payback period compared with un-subsided gas 3.1 years.

It is worth to mention that the current natural gas prices have been used for calculation of Payback period. However, during last two years natural gas prices have been increased more than 70%. Keeping in view the above trend of price rise in natural gas, this will further reduce the Payback period. The normal life of solar

dryer is around 25 years. Therefore, solar dryer is economically feasible in Pakistan in comparison to all sources of energy, as we have compared solar drying with cheapest source of energy i.e. natural gas even if, it is available in area. In case of non availability of the natural gas the cost of transportation would further increase the cost of conventional energy.

Reasons for unacceptability of solar energy in Pakistan. Though solar thermal technology is economically viable option in Pakistan but still it is not socio-economically widely acceptable for number of following reasons which were observed during this study.

- A large population of Pakistan, especially farmers, is illiterate and they are unaware of solar thermal echnology, its use, feasibility and benefits. Unfortunately, solar thermal technology is normally compared with photovoltaic energy which is unjustified.
- Solar energy is abundant in Pakistan but the expertise are lacking for system sizing in solar thermal energy technologies to perfectly meet the needs of the farmers/customers. As a result few unsuccessful stories by untrained technician defamed the solar thermal energy technologies.
- In very near past, natural gas, electricity and petroleum products were subsided in Pakistan for farmers and renewable energy was not on the priority list of Government of Pakistan. But now the above mentioned subsidies have been withdrawn and farmers are looking for alternative options. However, it will take time for solar thermal technology to penetrate in the market as communication medium in remote areas are not very effective compared to the cities.
- Public sector organization like Pakistan Council of Renewable Energy Technology and Alternative Energy Development Board, responsible for promotion and dissemination of renewable technologies in the country are not efficient and also do not have the capacity to reach remote areas thoroughly. The linkages among various such organization and other stakeholders like farmers and local governments are very weak Shah *et al*. (2011). As a result the dissemination of solar thermal technologies like dryers is limited to few places only.
- The policies of government of Pakistan in the past were not encouraging for promotion of renewable energy technologies. However, recent sever energy crises of Pakistan forced policy makers to promote renewable technologies especially solar thermal technologies as an alternative source of energy to reduce the energy shortfall in the country. As a result Government of Pakistan showed commitment to promote solar thermal technologies through tax exemptions and establishment of pilot projects in solar thermal technologies and this project is one such example.
- Socially farmers are reluctant to leave traditional techniques of fruit and vegetable dryings. A campaign to familiarize solar technologies through electronic and print media with the cooperation of local government is essential for its success.

Conclusions

This solar dryers design is successful for medium and large size Dates growers and it is cost effective to dry Dates in sunny climates for preservation of entire next year. The quality of the Dates dried in controlled environment was very good for human consumption. This dryer is capable of drying other fruit and vegetables with slight modification in air flow rate. This study also envisages that the services of only trained technician for system sizing for solar dryers must be used to avoid perception of technology failure. The close coordination among organization disseminating solar thermal technologies and its users is essential. Subsidized policies for renewable energy technologies should be adopted for at least ten years to ensure consistency in subsidy policies. A crash awareness program in solar thermal technologies should be launched in local languages which are understandable to the farmers.

Conflict of Interest. The authors declare no conflict of interest.

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Pak. j. sci. ind. res. Ser. B: biol. sci. 2021 **64B**(1) 38-42

Elemental Profile of Kinnow (*Citrus reticulata*) Growing in Sargodha District

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(received April 19, 2017; revised December 24, 2018; accepted January 17, 2019)

Abstract. Citrus is one of the most commonly used as a fruit in Pakistan. To estimate their elemental profile citrus were collected from five different tehsils of Sargodha district. Elemental analysis was carried out by atomic absorption spectrophotometer. There results showed that citrus fruits have riched source of minerals and have highest concentrations of Cu (2.71 mg/Kg), Mn (0.1633 mg/Kg) and Zn (31.72 mg/Kg) noted in T1 (Silanwali), while the maximum concentrations of Mg (65.247 mg/Kg), Cr (0.343 mg/Kg), P (1.146 mg/Kg), Co (0.536 mg/Kg), K (133.01 mg/Kg) were found in T2 (Sahiwal), T4 (Kotmomin) and T5 (Bhehra), respectively. Fluctuation in elemental profile of *Citrus reticulata* may be attributed to spatial variations or may be due to agro-climatic conditions that varies in all tehsils.

Keywords: elemental profile, *Citrus reticulata*, district Sargodha

Introduction

Citrus reticulata (Rutaceae) fruits are in Pakistan's most common edible food (Swingle, 1967) and are distributed in worldwide in temperate and tropical regions, mainly on the continents of Southern Africa and Australia (Parveen *et al*., 2015).

Citrus is grown throughout Pakistan, but the large area under cultivation of this tree is in Punjab provice (Jaskani and Abbas, 2007). Punjab's Kinnow and Feutrell grow two varieties of citrus covering the 80% of the total citrus area. The field of Sargodha and Faisalabad divisions are popularly known as Pakistan's California. Citrus fruits have rich source of ascorbic acid and not only important for domestic consumption (Ghafoor *et al*., 2008). Citrus fruits are grown in Pakistan over an area of 183.8 thousand hectares with a total production of 1,943.7 thousand tonnes and currently 94,806 tonnes of citrus (Kinnow and others) fruits are exported to various countries (Altaf *et al*., 2008).

Minerals are essential for plants such as potassium increases the activity of the enzymatic reaction, also involves stomata control (opening and closing of stomata), helps the plant from adverse and harsh conditions such as flooding and drought, the maximum potassium concentration in chloroplast is present and cytosol may cause soluble and often non-soluble large molecules to be neutralised (Marschner *et al*., 1990). While, magnesium plays a vital role in the formation of chlorophyll and plays very important role in photosynthesis, without the magnesium, the plants do not grow very well, it helping in green light absorbing pigments to catch the light energy and converting it into chemical energy for the development of lipids and proteins, carbohydrates (Cakmak *et al*., 2010), it also plays important role in oxidation reduction processes and also helps in the electron transport chain, which is very important and essential for the photo system II, also causes the regulation of enzymes (Mousavi, 2011). Zinc is also an essential element for the formation of tryptophan and is major component of some enzymes like dehydrogenesis and involves in the metabolic process (Nagarajan *et al*., 2014). Zn deficiency causes the plants to have intermediate chlorosis. It causes the leaves to bend and changes the colour of leaves. The reduction in fruit formation, stunted growth and chlorotic lesions are also caused by deficiency (Chatterjee *et al*., 2018).

Minerals are important for our health and survival (Ghani *et al*., 2017a), in human body, 5% of the body mass is consisted of mineral matter (Alexander *et al*., 2008). As a part of hamoglobin myoglobin, it is also essential for our nervous system to severe as a trigger for many biological reactions (transmission of messages from one part of body to another), for proper food digestion (Ghani *et al*., 2017b), metabolism, utilization

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of all nutrients in food (Alexander *et al*., 2008). i.e. Mg for vitamin 'B' utilization, Zn for vitamin 'A', Se for vitamin 'E' absorption, Ca for ascorbic acid, also important for maintaining acid alkaline balance in our body, they are also essential for our heart proper functioning, acting as electrolytes in our body (Hall, 2015). Therefore, the purpose of the study is to find out the spatial variation in the elemental profile of *Citrus reticulata* rising in the Sargodha district's various tehsils.

Material and Method

Sample collection. *Citrus reticulata* were collected from five tehsils (Silanwali T1, Sahiwal T2, Bhalwal T3, Kotmomin T4 and Bhera T5) of Sargodha district, Punjab for elemental analysis with three replicates. Each sample was handpicked randomly, wrapped in a special brown envelope and numbered. All the samples were dried in oven fir 3 days at a temperature of 75 °C.

Sample preparation for AAS analysis. The oven dried fruit samples were grinded into fine powder and then digested by wet digestion method. 0.5 g of each sample was taken into the digestion flask with 10 mL HNO_3 acid and kept them for overnight. Preparation of standards as well as subsequent samples were carried out as per AOAC (1998) methods.

Statistical analysis. Statistical analysis was carried out using Microsoft Excel 2007 (Steel *et al*., 1997).

Results and Discussion

Results in Table 1 findings showd that *Citrus reticulata* cobalt content ranged from 0.536 mg/Kg (T5) to 0.156 mg/Kg (T4) with the mean value of 0.300 mg/Kg, while Cr range from 0.343 mg/Kg (T4) to 0.03 mg/Kg (T3) with the mean value of 0.114 mg/Kg. The Cu value ranged from 2.71 mg/Kg (T1) to 2.036 mg/Kg (T5) with an average of 2.409 mg/Kg, while K value ranged from 133.013 mg/Kg (T5) to 110.806 mg/Kg (T3) with an average value of 120.66 mg/Kg. The variation in Mg content ranged from 65.247 mg/Kg (T2) to 53.63 mg/Kg (T5) with the mean value of 59.43 mg/Kg. Mn was found to be highest 0.163 mg/Kg in (T1) and lowest 0.051 mg/Kg in (T4) with the mean value of 0.084 mg/Kg. The P value ranged from 1.146 mg/Kg (T4) to 0.153 mg/Kg (T3) with the mean value of 0.717 mg/Kg, while the Zn - value ranged from 31.726 mg/Kg (T1) to 26.463 mg/Kg (T5) with a mean of 29.75 mg/Kg.

Cobalt is an essential in our body as it is a component vitamin B12 and helpful in curing anemia is an essential improving the formation of RBCs, also good for nervous system, and our mental health. Its deficiency leads to anemia, mental disturbance, nerve disorders, vitamin B12 deficiency and abnormal cell formation (Dolara, 2014; Soetan *et al*., 2010). The National Institute for Occupational Safety and Health explains that the usual cobalt dosage is 0.05 mg/m^3, where as up to 0.1 mg/m^3 can be tolerated. A dose of 20 mg/m^3 is harmful to life and health (Barbera *et al*., 1989). Results of the present study regarding cobalt concentration were higher than the Salimpour *et al*. (2010) that varied between 0.015 to 0.046 mg/Kg in different samples of citrus, while in Basil and pumpkin ranged from 0.015-0.016 mg/Kg and 0.041-0.050 mg/Kg, respectively.

Chromium is essential for the metabolism of carbohydrates and which are important for proper functioning of brain and other body processes. It also supports glucose metabolism and function of insulin. This deficiency may contribute to diabetes (Mason, 2011). The standard chromium dose for women is suggested as 14-25 µg/day, while for men, ranged from 14-35 µg/day. Children's aged with age between 1-13 years

Table 1. Comparison of means regarding elemental profile in *Citrus reticulata*

Metals	Tehsils				
(mg/Kg)	T1	T2	T3	T4	T5
Co	0.27±0.06	0.267±0.04	0.273±0.03	0.156±0.02	0.536±0.43
Cr	0.066±0.01	0.073±0.01	0.03±0.01	0.343±0.26	0.06±0.02
Zn	31.726±1.31	30.146±1.21	31.59±1.12	28.846±1.89	26.463±1.57
Mn	0.163±0.05	0.081±0.007	0.067±0.012	0.051±0.01	0.061±0.01
Mg	59.97±1.20	65.247±1.92	62.03±1.02	56.26±1.98	53.63±1.84
K	130.516±2.78	112.883±1.87	110.806±2.32	116.112±2.25	133.013±2.12
P	0.69±0.55	0.916±0.69	0.153±0.08	1.146±0.51	0.683±0.52
Cu	2.71±0.76	2.406±0.16	2.293±0.55	2.6±0.39	2.036±1.18

also need chromium ranged between 0.2 to 25 µg/day (Institute of Medicine, 2001). Results regarding chromium concentration in *Citrus reticulata* are different from the findings of Ihesinachi and Eresiya (2014). They noted that chromium concentration in orange fruit ranged from 46.2 mg/Kg to 84.7 mg/Kg.

Phosphorus is another important element in nature which plays an important role in the structure framework of DNA and RNA and necessary for cellular processes i.e. photosynthesis, phosphorylation, key component of ATP and NADPH (Institute of Medicine, 1997), main structural component of all cellular membranes (phospholipids) and also helpful in building of bones and teeth with calcium as a calcium phosphate (Nelson and Cox, 2000). An average adult human contains about 0.7 mg/Kg of phosphorus from which 80-90% present in the teeth and bones. Normal intake of phosphorus for adults and children's should not be exceeded from 580 mg/day and 460 mg/day is suggested by (Institute of Medicine, 1997). The present results regarding phosphorus are in collaboration with the findings of Salimpour *et al*., 2010. Who found that phosphorus varied in different samples of citrus ranged from 0.03 mg/Kg to 5.70 mg/Kg, while (Ihesinachi and Eresiya, 2014) described showed that level of phosphorus in citrus ranged from 30.0 mg/Kg to 61.5 mg/Kg.

Zinc is another important metal that strengthens the body defense system, also plays an important role in cell division, cell development and hypogonadism weak appetite, odour and taste problems. Slow growth, skin sores, dark problems, injuries and cuts recover (heal) take more time. These signs of symptoms can removed within a small period of time after intake of zinc, enrich food or supplements. (Mason, 2011; Institute of Medicine, 2001). Adequate intake of zinc is also suggested by Institute of Medicine (2001). The standard dose of zinc is suggested as 2 mg/day to 8 mg/day, while for adults is suggested as 8 mg/day to 13 mg/day. Present results regarding zinc concentration are collaborating with the findings of (Ihesinachi and Eresiya, 2014). In various citrus samples, its level varies from 1.10 mg/Kg to 30.23 mg/Kg.

Copper is an important element that acts as a catalyst in the body and also important for cell physiology, respiration, free radical scavenging, elastin cross-linking, oxidative defense system, needed for body pigmentation, maintain a healthy CNS, prevents anemia, also keeps immune system, vessels and bones healthy (Mason, 2011), while adequate copper intake of is suggested as ranging from 200 µg/day to 220 µg/day. For adults it is suggested as 340 µg/day to 1,300 µg/day (Institute of Medicine, 2011). The results of the copper concentrations are in collaborating with the results of (Ghani *et al*., 2017b). Copper content ranges from 4.043 mg/Kg to 1.403 mg/Kg, while (Dhiman *et al*., 2011) described that concentration of copper was 5.90±0.075 mg/Kg in citrus fruits.

Magnesium plays important role in metabolism, as a co-factor and several enzymes plays an essential role in metabolism which is also important in glucose metabolism, proper muscle functions and immune system, keep bones strong and healthy, regulate glucose level in blood, help in ATP synthesis and also important for proper functioning of heart (Mason, 2011). Adequate intake of magnesium for infants ranged from 30 mg/day to 240 mg/day but for adults it can be tolerated up to 400 mg/day (Institute of Medicine, 2001). Present results of the magnesium concentration are higher than the findings of (Osarumwense *et al*., 2013). Their ranges are from 15.55±1.45 mg/Kg to 21.87±2.39 mg/Kg in different samples of citrus.

Manganese is a mineral which is helpful for body in formation of sex hormones, blood clotting factors, connective tissues, blood sugar regulation, calcium absorption and also component of different enzymes (Soetan *et al*., 2010). It plays important role in delay of aging, improve health conditions including heart diseases and cancer (Aschner and Aschner, 2005). Adequate intake of manganese for infants ranged from 1.2 mg/day to 1.6 mg/day but for children of age 9 to 14, it can be tolerated up to 2.2 mg/day. However, for adults it can be tolerated up to 2.3 mg/day (Institute of Medicine, 2001). Our results regarding manganese concentration are in collaboration with the findings of (Ghani *et al*., 2017b), who found Mn varied from 0.226 mg/Kg to 0.153 mg/Kg in different samples of citrus.

Potassium is also an essential mineral for the maintenance of several processes in our body (proper functioning of cells, tissues and organs), acting as an electrolyte in the body (Ghani *et al*., 2017b), essential for proper heart functioning (regulating heart beat), also helpful in food digestion and muscle contraction (Hermansen, 2000), while adequate intakes potassium are 400 mg/day to 700 mg/day for infants and up to 3,000 mg/day to 4,500 mg/day for children of age up to 14 years. However, for adults it may be tolerated up

Table 2. Comparison of highest elemental profile in *Citrus reticulata* at different tehsils of Sargodha district

Metals (mg/Kg)	Tehsils				
	T1	T2	T3	T4	T5
Mg		✓			
Mn	✓				
Zn	✓				
Cr				✓	
Co					✓
K					✓
P				✓	
Cu	✓				
Total	3	1	0	2	2

to 4,700 mg/day (Institute of Medicine, 2011). Present results of potassium are in collaboration with the findings of Ghani *et al*. (2017b). The potassium content ranges from 40.681 mg/Kg to 173.42 mg/Kg with the mean value of 95.7412 mg/Kg.

Conclusion

All tehsils have good source of components, but *Citrus reticulata* of tehsil Silanwali (T1) contains the highest elemental profile as shown in Table 2. Fluctuation in the elemental profile of *Citrus reticulata* present in all tehsils may be attributed to environmental factors (soil composition, water, temperature and light), time of fruit harvest or may be due to spatial variations.

Conflict of Interest. The authors declare no conflict of interest.

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Pak. j. sci. ind. res. Ser. B: biol. sci. 2021 **64B**(1) 43-48

Integration of Phosphate Solubilising Bacteria, Sulphur Oxidizing Bacteria with NPK on Maize (*Zea mays*)

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(received March 27, 2019; revised March 20, 2019; accepted March 21, 2019)

Abstract. Deficiency of phosphorus can reduce the yield up to 15-20% and its availability to crop plants is the main issue, particularly in calcareous soils of Pakistan. Micro-organisms, phosphorus solubilizing bacteria (PSB) and sulphur oxidizing bacteria (SOB) have the ability to make P accessible for optimum plant growth, under conditions of nutrients disparity. Therefore, six treatments *i.e.* control, ½ dose of NPK, full recommended dose of NPK, ½ NPK+ SOB, ½ NPK + PSB and ½ NPK + SOB+ PSB were applied in a field experiment to investigate the integrated role of SOB and PSB with NPK fertilizers for enhancing the maize production. The treatment was concluded to have major impact on agro-morphological traits, seed quality and growth parameters of maize. Results depicted that the use of ½ NPK + SOB + PSB gave maximum germination count/plot (151.33), plant height (189.03 cm), number of ears/plant (1.60), grains/ear (472.33), 1000-grain weight (305.67 g), grain yield (5350.50 Kg/ha), harvest index (31.23%), leaf area (379.77 cm^2), total dry matter accumulated (181.43 g/plant), crop growth rate (30.60 g/day), net assimilation rate (9.31 g/day) and protein contents (8.49%).

Keywords: maize, PSB, SOB, NPK, growth, quality parameters

Introduction

Maize (*Zea mays* L.) has an important role in Pakistan's current cropping scheme and ranks third after wheat and rice (Farhad *et al*., 2009). Maize is one of the major export crops and with great economic and social importance has an important role in human and animal nutition. It contributes 2.1% in agriculture and 0.4% in gross domestic products (Chandio *et al*., 2016). The growing demand for increased yield in agriculture, also grows the demand for new technologies with less impact on natural resources. The use of micro-organisms that promoter plant growth can suport or even satisfy the demand for nitrogen (N) and phosphorus (P) in different crops (Baldotto *et al*., 2010; Baldani *et al*., 2009).

Phosphorus (P) is a most important growth limiting nutrient (Sharma *et al*., 2013) and plays important role in nearly all phases of plant cycle including root growth, photosynthesis, anthesis, seed production and maturation. Its deficiency causes stunted growth and severe yield losses. Its concentration in soil solution is very low, because soluble forms of P are fixed by soil solid phase, making less than 0.01% of total P available to plants (Niazi *et al*., 2015). Therefore, one phosphorous is one of the least mobile nutrients in soil (Balemi and Negisho, 2012). Most of the P that is present in soil and supplied to crops by inorganic fertilizers becomes unavailable by precipitation by reacting in acidic soils with Fe^{+3} and Al^{+3} and with Ca^{2+} in calcareous soils, respectively (Abbasi *et al*., 2015). Different genera of bacteria have the ability to mineralize and solubilize P pools in soil and these bacteria significantly promot their bioavilability. This process not only compensates the input of high cost fertilizers but it can also enhance the mobilisation of insoluble P already added to soil from the fertilizers. Such group of bacteria is termed as phosphate solubilizing bacteria (PSB) and inoculation with PSB as biofertilizers enhances P accumulation and biomass production of plants (Abbasi *et al*., 2015). Phosphates solubilizing bacterial (PSB) species have capacity to mineralize the both natural and inorganic (p) phosphorus (Khiari and Parent, 2005). Phosphorus accessibility from the soil can be extended by phosphate solubilizing bacteria (PSB) with microscopic organisms oxidi-sing sulphur studied by (Turan *et al*., 2007). The immunization of phosphorus solubilizing micro-organisms (PSB) enhanced the yield of crop by solubilizing the phosphate that was associated on soil and stable in soil Gull *et al*. (2004).

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A field survey was directed to determine the influences and impact of (PSB) and diverse levels of (P) fertilizer on yield and quantities of the crop by Sial *et al*. (2015). Diverse microbial inoculums levels were utilized alone and joined with phosphorus fertilizer. Uzma *et al*. (2014) concluded that the levels of inorganic fertilizers from 60-100% and recommended dose of NPKZn increased the cob^{-1} length, 1000-grain weight, grain protein and starch content of maize. (Azotobacter+PSB) showed similar results when dual inoculation applied to the seed. Abbas *et al*. (2013) resulted that the co-inoculation of PGPR and PSB indicated maximum plant height. The treatment with the combination of Iple Iple (II) + PSB + recommended K + ¾ N + ¾ P gave maximum plant phosphorous content. Amanullah and Khan (2015) concluded that phosphorous applied at the higher rates and compost applied at sowing time increased maize yield and maize yield components significantly. The yield and yield components of maize were strongly increased under semi aride conditions with maize seeds. Baloach *et al*. (2014) were studied the combine effects of PSB and humic compounds to improve the yield of maize. The humic acid at 10 Kg/ha + PSB bio-fertilizer at 2 Kg/ha showed maximum results in that parameters biological yield, harvesting index, grain yield and stover yield as equated to control. Amanullah *et al*. (2010) studied the effect of phosphorus on maize yield and growth. Phosphorus has a significant impact on crop growth rate (CGR), dry matter (DM), leaf area index (LAI) and the biological yield from leaf area ratio (LAR), relative growth rate (RGR), net assimilation rate (NAR) has no important effect.(NAR), absolute growth rate (AGR) and (RGR) show the negative effect due to increasing the plant density. Realizing the importance of PSB and SOB in an integrated manner with NPK for improving the nutrients availability especially P, a field experiment was executed to investigate the initial growth and mineral nutrition of maize (*Zea mays* L.) in response to application of NPK rates combined with the inoculation PSB and SOB.

Materials and Methods

A field experiment was conducted at National Agriculture Research Centre, Islamabad (Maize Program) during the autumn, 2016. The treatments were arranged in Randomized Complete Block Design (RCBD) with three replications. Seeds of maize Cv. Islamabad Gold were sown on ridges maintaining. The treatments were controled (without fertilizer), ½ dose of NPK, full recommended dose of NPK, ½ NPK + SOB, ½ NPK + PSB and ½ NPK + SOB+ PSB. PSB and SOB are collected from the microbiology laboratory, Land Resources Research Institute, National Agricultural Research Centre, Islamabad. Maize seeds were inoculated with phosphorus solubilising bacteria (PSB) and sulphur oxidizing bacteria (SOB). Data were collected on growth and yield chrematistics; germination count/plot, Plant height in (cm), ear number/plant, grain/ear, 1000-grain weight (g), grain yield (Kg/ha), harvest index (%), leaf area (cm^2), total dry matter accumulation/ plant (g), crop growth rate (g/m^2/day), net assimilation rate (g/m^2/ day) and grain protein contents (%). Data collected were subjected to analysis of variance and the means obtained was compared by LSD at 5% level of probability (Montgomery, 2001).

Results and Discussions

Statistical analysis of the maize germination count revealed a substantial difference between various treatments (Table 1). The maximum germination count was recorded (151.33 plants/plot) by the application of ½ NPK + PSB + SOB, which was statistically equal to

Table 1. Effect of NPK with PSB and SOB on agro-morphological, growth and quality parameters of maize

Treatments	Germination count/plot	Plant height (cm)	Ear number/ plant	Grain/ Ear	1000-grain weight (g)	Grain yield (Kg/ha)	Harvest index (%)	Leaf area (cm^2)	Total Dry matter accumulation/ plant (g)	CGR/g/ day	NAR/g/ day
Control (T_1)	79f	155e	1.07 c	261e	280f	3114f	23f	199f	151f	27e	4c
½ NPK(T_2)	95e	166d	1.27abc	298d	285e	4114e	26e	218e	159e	28d	5c
Full NPK(T_3)	114d	172c	1.20bc	334c	290d	4324d	27d	244d	163d	28d	6b
½ NPK+PSB(T_4)	127c	78b	1.33abc	352c	293c	45324c	29c	302c	171c	29c	7b
½ NPK+SOB(T_5)	140b	182b	1.47ab	429b	297b	4739b	30b	326b	174b	29c	7b
½ NPK+PSB+SOB(T_6)	151a	189a	1.60a	472a	306a	5022a	31a	380a	181a	31a	9a
LSD	6	5	0. 5	21	3	7	1	14	3	0.2	1

Means with different letters are significantly different at 5% level of probability.

½ NPK + SOB (140.67) and ½ NPK + PSB (127.33), followed by complete NPK (114.33 plants/plot) and ½ NPK (95.00 per plot), while the minimum germination was recorded in control treatment (79.00 plants/plot). The results are in confirmatory with Hameeda *et al*., (2008) who described that application of PSB enhanced the germination of maize. Phosphate Solubilizing Bacteria (PSB), which are rhizobacteria that convert insoluble phosphates into soluble forms through acidification, chelation, exchange reactions and production of organic acids.

The maximum plant height (189.03 cm) was recorded in ½ NPK+phosphorous solubilizing bacteria and sulphur oxidizing bacteria followed by treatment ½ NPK + SOB) (182.07) which was found statistically at par with ½ NPK + PSB) (177.90). However, the minimum plant height (155.37 cm) was measured in control. The data reported showed that the application of PSB, SOB or both bio-fertilisers by soil application methods increased height of the plant (Table 1). Experimental findings are in confirmatory with Shafiq and Tahir in (2015).Similar results are also reported by Abbas *et al*. (2013). Release of P by PSB from insoluble and fixed/ adsorbed forms is an import aspect regarding P availability in soils. There are strong evidences that soil bacteria are capable of transforming soil P to the forms available to plant. Microbial biomass assimilates soluble P, and prevents it from adsorption or fixation (Khan and Jorgensen, 2009).

The maximum number of ear/plant (1.60) was observed in ½ NPK + PSB + SOB treatment which are statistically at par with ½ NPK + SOB treatment (1.4). The data inferences that ear/plant (Table 1) showed non-significant response of different treatments.. The minimum number of ear/plant (1.06) was taken in treatment (control), whereas T_1, is statistically at par with T_2, ½ NPK (1.27) and treatment T_3, full NPK (1.20) and T4, (1.33) are also statistically at par with each other. Significant difference was found among various treatments on grain/ear (Table 1). The results showed that in treatment, the highest grain/ear (472.33) was counted when ½ NPK + PSB + SOB was applied followed by ½ NPK + SOB (429.33) and treatment ½ NPK + PSB(351.67), which was statistically at par with complete NPK (334.33). It was also cleared from the data that the lowest grain/plant were recorded with T_1 (261.33) followed by T_2 (298.33) respectively. Our findings are similar with Asghar *et al*. (2010), our results are closely in line with Jinjala *et al*. (2016).

1000-grains weight of maize crop was analyzed that significant difference among treatment means shown in (Table 1). The plot which we apply, ½ NPK + PSB + SOB showed maximum 1000-grains weight (305.67) followed by T_5 (297.50), T_4 (293.10), T_3 (289.60) and T_2 (285.10) respectively, whereas the minimum 1000-grain weight (280.33) was detected in that plot which was not use any fertilization T_1 (control). Maize grain weight was increased by the application of NPK due to increased nutrient efficiency. Our findings are in line with Uzma *et al*. (2014). The similar results are also found with Amanullah and Khan (2015).

The statistical data indicated that grain yield was recorded in treatment (Table 1). The maximum grain yield (5022.4 Kg/ha) was observed in the treatment ½ NPK+ PSB + SOB were applied. Followed by T_5 (4739.3 Kg/ha), T_4 (4532.4 Kg/ha), T_3 (4324.7 Kg/ha) and T_2 (3914.4 Kg/ha) respectively. The treatment T_1, (control) shown minimum grain yield (2114.3 Kg/ha). The related results are initiated with Upadhyay *et al*. (2016). Similar judgments were also reported by Baloach *et al*. (2014). The yield is calculated from the 1000 grain weight so, the increasing the grain yield. As we see the significant differences between the weight of 1000 grain and grain yield which are the same. The results are in confirmatory with Amanullah and Khalid (2005) and Singh *et al*. (2004).

Data on the harvest index revealed a substantial difference between the means of treatment (Table 1). The maximum harvest index (31.22) was observed in that plot which we apply both phosphorous solubilizing bacteria and sulphur oxidising bacteria T_6, followed by ½ NPK+SOB treatment T_5 (29.77), T_4 (28.59), T_3 (27.47) and T_2 (25.70), respectively. Whereas, in the plot there is no use of fertilizer (T_1) and which was showed minimum value of control of harvest index (23.45). Results seen indicated in line with Amanullah and Khan (2015). Our findings are similar with Baloach *et al*. (2014).

Data regarding leaf area per plant showed a significant difference between the treatments (Table 1). It was concluded that maximum leaf area/plant (LAP) (379.77 cm^2) was found in treatment, where ½ NPK+PSB+SOB (T_6) was applied. Followed by the treatment (T_5) ½NPK + SOB give (325.77 cm^2), (T_4) ½ NPK + PSB (302.30 cm^2), (T_3) Full NPK (244.20 cm^2) and (T_2) ½ NPK (218.37 cm^2) respectively. However, minimum leaf area/plant (199.33 cm^2) was measured in control treatment (T_1).The results are in confirmatory with the

Banerjee *et al*. (2006).Who concluded that N120SSP30-VAM and N120RP30PSB gave higher yield and yield contributing attributes. The similar results were reported Nwanyanwu *et al*. (2015).

Results inference that maximum dry matter/plant was initiate in treatment (T_6), where ½ NPK + SOB + PSB was applied (181.43 g). The following treatments (174.23 g), (171.0 4 g), (162.87g),(159.53 g) are ½ NPK + SOB (T_5), ½ NPK + PSB (T_4), full NPK (T_3) and ½ NPK (T_2) statistical analysis indicated that significant difference among the treatment means shown in (Table 1). However, the treatment (T_1) minimum dry matter/plant were measured in control. Our results are in line with Banerjee *et al*. (2006).

The maximum crop growth rate (CGR) (30.60 g/day) was observed in crop plant which we applied the treatment T_6, ½ NPK+PSB+SOB followed by T_5 (29.30/ g^2/day), T_4 (28.79/ g^2/day) data concerning crop growth rate indicated significant difference among the treatment means (Table 1). T_3 (28.42/g^2/day) are statistically at par with T_2 (28.26/g^2/day). On the other hand the minimum value of CGR (27.16/g^2/day) was observed in treatment T_3 (control) in which no synthetic and bio fertilizer are used. The results are comparable with Banerjee *et al*. (2006).

Net assimilation rate (NAR) of maize crop was inclined by the application of phosphorus solubilizing bacteria and sulphur oxidizing bacteria through soil application and inferences statistically significant difference (Table 1). T_5 (7.10) are at par with T_4 (6.82) and T_3 (6.50).Results indicated that maximum (NAR) was found in treatment T_1 (9.31), where (½ NPK+PSB+SOB) was applied. The minimum value was found in control treatment T_1 (1.71) which is statistically similar with T_2 (4.44). The study results are in coherent with Amanullah *et al*. (2010).

Analysis of variance showed the statistically significant effect of both PSB and SOB along with ½ NPK on grain proteins content (Table 1).Result depicted that maximum grain proteins content was analyzed in the treatment T_6 (8.49), where ½ NPK, phosphorous solubilizing bacteria and sulphur oxidizing bacteria was applied. Followed by T_5 (7.08), T_4 (6.89), T_3 (6.17) and T_2 (5.56) respectively. The minimum protein content was recorded in the control of treatment T_1 (4.85), and this may be ascribed to intense protein synthesis in plants and its efficient storage in the presence of abundant supply of available nutrients through bio-fertilizer and organics. The easy availability of nutrients leads to balanced C: N ratio which enhanced the vegetative growth of plant resulting in high photosynthetic activity. Which finally out yielded better protein content in plant and higher grain yield which in turn improved the protein yield. The results of present investigation corroborate with the findings of few previous studies (Sharma *et al*., 2013a and b; Pathak *et al*., 2002). Findings of this study are similar with Shafiq and Tahir (2015) who was reported increase in grain proteins with the application of (PSB) *Bacillus subtilis* and *Vesicular arbuscular* mycorrhiza (VAM). Similar findings were also conveyed by Jinjala *et al*. (2016).

Conclusion

Conjunction of NPK with PSB and SOB improved maize growth and the yield and quality. Bio-fertilizers could save the environment and improve the economics of maize growers.

Conflict of Interest. The authors declare no conflict of interest.

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Pak. j. sci. ind. res. Ser. B: biol. sci. 2021 **64B**(1) 49-54

Growth, Yield and Quality Response of Three Wheat Varieties to Foliar Spray of Micro Nutrients

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(received April 9, 2019; revised September 17, 2019; accepted September 19, 2019)

Abstract. A field experiment was carried out to test the result of foliar application of micro nutrients on growth, quality and yield of three wheat verities. The trial was performed at Agronomic research area, University of Agriculture Faisalabad. Wheat varieties Jouhar 2016, Ujala 2015 and Gold 2016 were sown. Combination of micro nutrient (Zn 2%, B 0.5%, Fe 1%, Mn 1% and Cu 0.5%) with different amounts (no spray, water spray, 1000 mL/ha, 1250 mL/ha and 1500 mL/ha) was sprayed at booting stage. By results of this study,it is concluded that foliar spray of micro nutrients has significant effect on growth, yield and quality parameters. Statistically highly significant effect of 1250 mL/ha spray of micro nutrients mixture was observed on plant height (105.33 cm), grains/spike (48), grain yield (5336.3 Kg/ha), biological yield (12829 Kg/ha), carbohydrates (63.7%) and protein (11.4%) under V_2F_4 (Ujala 2015 + 1250 mL/ha micro nutrients).

Keywords: wheat varieties, foliar application, Zn, Fe, Mn and Cu

Introduction

Wheat is an important cereal crop of Pakistan. It is sown worldwide on wide range of different climatic conditions as compared to the other grain crops. Wheat is rich source of protein, vitamins, minerals and carbohydrates (Habib, 2009). Wheat contribution in diet is about 70-72% (Ali *et al*., 2013; Habbasha *et al*., 2013). Micro nutrients deficiency in world population is more than 40% (Faraji *et al*., 2014; Abbas *et al*., 2011). Growth, development and yield of wheat crop decreased due to deficiency of micro nutrients (Nadim *et al*., 2012; Cisse and Amar, 2000). In Pakistan about 1980 thousand hectares is wheat cultivated area and production is about 25.48 million tons. Agriculture's share in GDP is 18.9 % and out of which 9.1 % is contributed by wheat (Govt. of Pakistan, 2018). Different factors trigger yield losses like late sowing, non-judicious use of fertilizer, less irrigation, excess of weeds in fields and drought for long time. (Zeidan *et al*., 2010). These factors are responsible in various stages of crop and harmfully impact the crop yield. For various crops, micro and macro nutrients deficiencies have been examined (Hussain *et al*., 2006). To regulate different metabolic processes and yield, each micronutrient plays a vital role. Zinc, Boron, Iron, Copper, Manganese and Molybdenum are important for plant's optimum growth and development (Rawashdeh and Florin, 2015; Zain *et al*., 2015). Boron is most essential for reproductive parts, cell multiplication, cell stabilization, carbohydrate utilization and formation of cell wall in plants (Biswas *et al*., 2015; Khan *et al*., 2010). Fe functions in chlorophyll biosynthesis,respiration, chloroplast development, carbohydrate productions, enzyme activation, biological redox system and act as oxygen carrier in nitrogen fixation (Rasul *et al*., 2015). Manganese is compulsory for metabolic reactions, accelerates chlorophyll biosynthesis, enzyme activation, electron transport, progresses immune system, improves uptake of Ca and P and assists in photosynthesis (Abbas *et al*., 2011). Copper performs numerous metabolic processes, aids in physiological redox processes, lignification and improves the uptake of N and interfaces with the other micro nutrients (Monreal *et al*., 2015; Habbasha *et al*., 2013). Increase in crop production cause the micro nutrients deficiencies (Fageria *et al*., 2002). Pakistani soils have deficiency of different micro nutrients such as boron deficiency is about 51-60% (Rashid *et al*., 2002). Wheat production can be enhanced by enhancing the yield per unit area. Plants require proper amounts of macro nutrients and micro nutrients for better growth.

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Materials and Methods

A field trial was conducted at Agronomic Farm University of Agriculture, Faisalabad during growing season 2016-17. Texture of soil was sandy loam. An

experiment was laid out in randomized complete block design with factorial arrangements and three replications. Plot size was 6.0 m long x 2.25 m wide. Treatments were different wheat varieties: V_1=Jouhar 2016; V_2=Ujala 2015; V_3=Gold 2016 and foliar applications: F_1=control(no spray); F_2= water spray; F_3= 1000 mL/ micro nutrients (Zn 4.7%, B 1.0%, Fe 2%, Mn 2%, and Cu 3%)/ ha F_4= 1250 mL micro nutrients (Zn 4.7%, B1.0%, Fe 2%, Mn 2%, and Cu 3%)/ ha and F_5= 1500 mL micro nutrients(Zn 4.7%, B 1.0%, Fe 2%, Mn 2%, and Cu 3%)/ha. Only single dose of spray was applied at booting stage. Recorded data was analysed statistically by Fisher's analysis of variance technique and treatments means were compared by using least significant difference (LSD) test at 5% level of probability (Steel *et al*., 1997).

Results and Discussion

Plant height (cm). The data showed that the plant height was significantly affected by varieties, foliar spray of micro nutrients and their interaction. The response of different wheat varieties and foliar application was found significant. The maximum plant height (105.33 cm) was obtained in treatment V_1F_4 (Jouhar 2016 + 1250 mL/ha micro nutrients). and the Minimum plant height (94.33 cm) was obtained in V_3F_1 (Gold 2016 + Control), however, it is statistically at par with treatments V_2F_1 (Ujala 2015 + Control) and V_3F_2 (Gold 2016 + Water spray). The findings of present study are corelated with Khan *et al*. (2010) which stated increased plant height by the micro nutrient's spray applied at different stages. Hussain *et al*. (2005) and Nadim *et al*. (2012) also described the similar results.

Number of tillers. Numbers of total and productive tillers are statistically non-significant for different wheat varieties, foliar application and their interaction.

Number of grains per spike. Statistically a greater number of grains per spike (48) was found under V_2F_4 (Ujala 2015 + 1250 mL/ha micro nutrients) followed by V_1F_4 (Jouhar 2016 + 1250 mL/ha micro nutrients) that produced 46 grains per spike. The lowest number of grains per spike (37) was in V_1F_1 (Jouhar 2016 + control) and V_3F_1 (Gold 2016 + control) that is statistically similar with V_2F_1 (Ujala 2015 + control), V_1F_2 (Jouhar 2016 + water spray) and V_3F_2 (Gold 2016 + water spray). Tahir *et al*. (2009) represented that foliage use of micro nutrients efficiently improved grains per spike. Micro nutrients mixture enhanced the grains per spike in wheat (Yaseen *et al*. 2011). Same results were described by Khan *et al*. (2010).

1000- grain weight (g). Treatment V2F4 (Ujala 2015 + 1250 mL/ha micro nutrients) gained significantly maximum 1000-grain weight (49 g). However, the lowest 1000-grain weight (41 g) was found under the treatment V_3F_1 (Gold 2016 + control) that is statistically at par with treatments V_1F_1 (Jouhar 2016 + control) and V_2F_1 (Ujala 2015 + control). The findings are corelated with Yaseen *et al*. (2010). The results are also considered by Kassab *et al*. (2004) and Torun *et al*. (2001) which stated that thousand grain weight in case of wheat was significantly increased by foliar application zinc.

Grain yield (Kg/ha). The treatment V2F4 (Ujala 2015 + 1250 mL/ha micro nutrients) produced statistically highest grain yield (5336.3 Kg/ha) followed by V_3F_4 (Gold 2016 + 1250 mL/ha micro nutrients). Whereas the lowest grain yield was showed under response of V_3F_1 (Gold 2016 + control) that is statistically same with treatments like as V_1F_1 (Jouhar 2016 + control), V_2F_1(Ujala 2015 + control) and V_3F_2 (Gold 2016 + qater spray). Yaseen *et al*. (2010) stated that foliar use of all the nutrients cause enhance 24-38% yield of the wheat crop.

Biological yield (Kg/ha). The treatment V_2F_4 (Ujala 2015 + 1250 mL/ha micro nutrients) produced maximum biological yield (12829 Kg/ha). The lowest biological yield (9946 Kg/ha) was accumulated in treatment V_3F_1 (Gold 2016 + control) that is statistical similar with treatments V1F1 (Jouhar 2016 + control), V_2F_1 (Ujala 2015 + control), V_1F_2 (Jouhar 2016 + water spray) and V_3F_2 (Gold 2016 + water spray) which produced 10686 Kg/ha, 10484 Kg/ha, 10583 Kg/ha and 10538 Kg/ha respectively. The conclusions are union with Khan *et al*. (2010) and Hussain *et al*. (2005) they stated that total biomass enhanced with the foliar nourishing of micro nutrients. The results are also in line with Esfandiari *et al*. (2016).

Straw yield (Kg/ha). Treatment V_2F_4 (Ujala 2015 + 1250 mL/ha micro nutrients) collected highest straw yield amount (7492.7 Kg/ha) that is statistically at par with treatments of V_2F_3 (Ujala 2015 + 1000 mL/ha micro nutrients), V_3F_4 (Gold 2016 + 1250 mL/ha), V_1F_4 (Jouhar 2016 + 1250 mL/ha), V_2F_5 (Ujala 2015 + 1500 mL/ha),V_3F_3 (Gold 2016 + 1000 mL/ha micro nutrients) and V_1F_3 (Jouhar 2016 + 1000 mL/ha micro nutrients).

However, the lowest straw yield (6222.7 Kg/ha) was exhibited in treatment V_3F_1 (Gold 2016 + control) that is statistically same with treatments V_1F_1 (Jouhar 2016 + control), V_2F_1 (Ujala 2015 + control), V_1F_2 (Jouhar 2016 + water spray), V_2F_2 (Ujala 2015 + water spray), V_3F_2 (Gold 2016 + water spray), V_1F_5 (Jouhar 2016 + 1500 mL/ha) and V_3F_5 (Gold 2016 + 1500 mL/ha).The findings of the current study are considered by El-Ghamry *et al*. (2009) who documented that micro-nutrients application improved straw yield in wheat. The same results are also presented by Ananda and Patil (2005).

Harvest index (%). Harvest index percent is the ratio of total biological yield to economic yield. Data revealed that regarding harvest index the maximum amount (41.65%) was observed in treatment V_2F_4 (Ujala 2015 + 1250 mL/ha micro nutrients) than all other treatments but this treatment effect was statistically not differ with V_1F_4 (Jouhar 2016 + 1250 mL/ha micro nutrients), V_3F_4 (Gold 2016 + 1250 mL/ha micro nutrients), V_1F_3 (Jouhar 2016 + 1000 mL/ha micro nutrients) and V_3F_3 (Gold 2016 + 1000 mL/ha micro nutrients). Whereas, the lowest harvest index percent (37.44%) was counted in treatment V_3F_1 (Gold 2016 + control) that is statistical at par with V_2F_1 (Ujala 2015 + control), V_1F_1 (Jouhar 2016 + control), V_1F_2 (Jouhar 2016 + water spray), V_2F_2 (Ujala 2015 + water spray) and V_2F_5 (Ujala 2015 + 1500 mL/ha micro nutrients). The findings of current study are correlated with Khan *et al*. (2010) as they have stated that harvest index enhanced with micro nutrients application. Same findings are also reported by Zain *et al*. (2015) too who stated that foliar use of micro nutrients increases the harvest index.

Protein contents (%). Treatment V_2F_4 (Ujala 2015 + 1250 mL/ha micro nutrients) statistically highest protein contents (11.4%). Minimum protein percentage (9.0%) was recorded under treatment of V_3F_1 (Gold 2016 + control) that is statistically similar with treatment V_1F_1 (Jouhar 2016 + control). Micro nutrients contributions in physiological procedures plants like amino acid

Table 1. Individual comparison of treatment means

Treatments	Plant height (cm)	No. of tillers/m	No. of productive tillers/m^2	No. of grains per Spike	1000-Seed weight
V_1-Jouhar-2016	102.80 A	306.27	41.60 AB	301.80	45.20 AB
V_2- Ujala-2015	98.73 B	307.00	43.00 A	304.27	45.86 A
V_3- Gold-2016	96.47 C	306.53	41.20 B	303.47	44.66 B
LSD value at 5%	0.82	NS	NS	1.48	0.72
F_1-Control	97.00 C	306.33	37.33 D	300.22	41.66 E
F_2-Water Spray	98.00 C	306.22	39.66 C	303.89	44.44 D
F_3- 1000 mL micro nutrients/ha	99.56 B	307.89	44.00 B	305.00	46.44 B
F_4- 1250 mL micro nutrients/ha	102.22 A	308.78	46.66 AB	305.78	48.22 A
F_5- 1500 mL micro nutrients/ha	99.89 B	303.78	46.67 A	301.00	45.44 C
LSD value at 5%	1.07	NS	NS	1.91	0.93
$V_1 \times F_1$	101.33 bc	306.00	37.00 g	293.00	41.66 fg
$V_1 \times F2$	101.67 b	307.00	39.00 e-g	305.00	45.00 e
$V_1 \times F3$	103.00 b	307.00	44.00 b-d	306.00	46.33 c-e
$V_1 \times F4$	105.33 a	307.00	46.00 ab	304.33	48.00 ab
$V_1 \times F5$	102.67 b	304.00	42.00 c-e	300.67	45.00 e
$V_2 \times F_1$	95.33 fg	306.00	38.00 fg	303.33	42.33 fg
$V_2 \times F_2$	97.33 e	306.00	41.00 d-f	303.67	45.33 e
$V_2 \times F_3$	99.33 d	307.33	45.00 a-c	304.67	47.00 b-d
$V_2 \times F_4$	102.00 b	311.00	48.00 a	307.67	49.00 a
$V_3 \times F_5$	99.67 cd	304.67	43.00 b-d	302.00	45.66 de
$V_3 \times F_1$	94.33 g	308.67	37.00 g	304.33	41.00 g
$V_3 \times F_2$	95.00fg	305.67	39.00 e-g	303.00	43.00 f
$V_3 \times F_3$	96.3 ef	307.67	43.00 b-d	304.33	46.00 de
$V_3 \times F_4$	99.33 d	308.33	46.00 ab	305.33	47.66 a-c
$V_3 \times F_5$	97.33 e	302.67	41.00 d-f	300.33	45.66 de
LSD value at 5%	1.85	NS	NS	3.31	1.60

Table 2. Individual comparison of treatment means

Treatments	Grain yield (Kg/ha)	Biological Yield (Kg/ha)	Straw yield (Kg/ha)	Harvest Index	Protein content (%)	Carbohydrates (%)
V_1-Jouhar-2016	4424.9 AB	11145 B	6719.6 B	39.61	10.04 B	62.12 B
V_2- Ujala-2015	4555.0 A	11624 A	7069.4 A	39.09	10.28 A	62.36 A
V_3- Gold-2016	4314.0 B	11057 B	6742.7 B	38.93	9.98 B	61.92 C
LSD value at 5%	153.08	354.19	257.31	NS	0.11	596.26
F_1-Control	3848.7 E	10205 D	6356.8 C	37.71 D	9.13 E	60.76 E
F_2-Water Spray	4065.6 D	10664 C	6598.BC	38.11 CD	9.54 D	61.20 D
F_3- 1000 mL micronutrients/ha	4755.2 B	11901 B	7145.9 A	39.97 B	10.53 B	62.96 B
F_4- 1250 mL micronutrients/ha	5171.1 A	12498 A	7326.6 A	41.37 A	11.03 A	63.40 A
F_5- 1500 mL micronutrients/ha	4316.0 C	11108 C	6792.0 B	38.89 BC	10.26 C	62.33 C
LSD value at 5%	197.62	457.26	332.18	1.13	0.14	769.76
$V_1 \times F_1$	3876.0 hi	10186 de	6310.0 e	38.06 ef	9.10 hi	60.80 gh
$V_1 \times F_2$	4070.0 gh	10583 de	6537.7 de	38.43 ef	9.40 fg	61.10fg
$V_1 \times F_3$	4745.0 c-e	11713 b	6967.7 a-d	40.54 a-d	10.50 cd	63.00 cd
$V_1 \times F_4$	5170.3 ab	12411 ab	7240.7 ab	41.57 ab	10.90 b	63.40 ab
$V_1 \times F_5$	4263.3 fg	10830 d	6567.0 c-e	39.38 c-f	10.30 de	62.30 e
$V_2 \times F_1$	3946.7 g-i	10484 de	6512.7 de	37.65 f	9.30 gh	60.90 gh
$V_2 \times F_2$	4113.3 gh	10870 cd	6756.7 b-e	37.84 ef	9.63 f	61.40 f
$V_2 \times F_3$	4884.0 b-d	12306 ab	7422.3 a	39.689 b-e	10.70 bc	63.10 bc
$V_2 \times F_4$	5336.3 a	12829 a	7492.7 a	41.65 a	11.40 a	63.70 a
$V_3 \times F_5$	4494.7 ef	11632 bc	7137.7 a-c	38.70 d-f	10.40 d	62.70 d
$V_3 \times F_1$	3723.3 i	9946 e	6222.7 e	37.44 f	9.00 i	60.60 h
$V_3 \times F_2$	4013.3 gi	10538 de	6524.7 de	38.07 ef	9.60 f	61.10 fg
$V_3 \times F_3$	4636.7 de	11684 b	7047.7 a-d	40.544 a-d	10.40 d	62.80 cd
$V_3 \times F_4$	5006.7 a-c	12253 ab	7246.3 ab	40.88 a-c	10.80 b	63.10 bc
$V_3 \times F_5$	4190.0 f-h	10862 cd	6672.3 b-e	38.58 ef	10.10 e	62.00 e
LSD value at 5%	342.29	792.00	575.36	0.87	0.25	1333.3

biosynthesis, activation of enzymes, and starch utilization, improved the accumulation of assimilates in seeds, which results in more protein contents in grain (Rasul *et al*., 2015; Khan *et al*., 2010).

Carbohydrate concentration (%). Carbohydrate concentration revealed that statistically significant effect of foliar response of wheat varieties on concentration of carbohydrates. Maximum concentration (63.7%) was found in treatment V_2F_4 (Ujala 2015 +1250 mL/ha micro nutrients) followed by V_3F_4 (Gold 2016 + 1250 mL/ha micro nutrients) that is obtained (63.1%) carbohydrates. On the other hand, the statistically lowest carbohydrates concentration was gathered under treatment V_3F_1 (Gold 2016 + control) that is statistically at par with treatments V_1F_1 (Jouhar 2106 + control) and V_2F_1 (Ujala 2015). The micro nutrient plays a vital role in several carbohydrate production, metabolic reactions, nitrogen fixation acts as an oxygen carrier, membrane integrity, starch utilization, in enzyme system acts as a co-factor and phytochrome activities (Monreal *et al*., 2015; Bameri *et al*., 2012).

Conclusion

This study concluded that foliar application of micro nutrients mixture (@ 1250 mL/ha) on wheat varieties produced higher yield and better quality of grains.

Conflict of Interest. The authors declare no conflict of interest

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Pak. j. sci. ind. res. Ser. B: biol. sci. 2021 **64B**(1) 55-63

Estimation of Heavy Metals and Associated Health Risk in Selected Vegetables Grown in Peri-Urban Areas of Multan and Rawalpindi, Pakistan

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(received January 8, 2018; revised April 2, 2018; accepted December 6, 2018)

Abstract. Food security is a serious issue in developing countries like Pakistan because of ever-increasing population. To feed the hunger population, safe and nutritious vegetables are growing concern as they are being polluted by heavy metals. The present study was conducted to investigate the concentration of heavy metals such as cadmium (Cd), chromium (Cr), lead (Pb), nickel (Ni), iron (Fe), manganese (Mn), and zinc (Zn) in highly consumed vegetable collected from peri-urban markets of Rawalpindi and Multan region. Health risk associated with the consumption of these vegetables in term of estimated daily intake of metals (EDIM) and health risk index (HRI) was also evaluated among local consumers. Results showed that mean values of cadmium (Cd), and lead (Pb) in all vegetable samples from both region were exceeding the respective MAL set by FAO/WHO. Estimated daily intake of heavy metals was found below than the permissible limit. EDIM showed following decreasing trend Fe > Zn > Mn > Ni > Pb > Cr and Cd, respectively. The health risk index (HRI) for all heavy metals were less than the threshold level (1), indicating no significant threat to the local population through the consumption of these vegetables.

Keywords: food safety, EDIM, HRI, Pakistan

Introduction

Vegetables are a vital part of our diet and are known as an inexpensive. Pakistan is producing a variety of vegetables to satisfy local as well as international demand. Being agro-based economy about 70% of our population is directly or indirectly associated with this sector. The total area under vegetable cultivation is about 2% of the total crop production, while the export share is very low 0.22% GOP (2013). Vegetables are very rich sources of important biochemical and nutrients like carotene, carbohydrates, calcium, iron, ascorbic acid and extensive concentration of trace minerals Jimoh and Oladiji (2005). These are the basic sources of energy, especially in poor countries. The minimum daily consumption of fruits and vegetables should be more than 400 g per person as described by World Health Organization (WHO) in order to get numerous nutrients for optimum health studied by Lock *et al*. (2005). In Pakistan per capita daily intake of vegetable is 134 g which is 66.5% below than the minimum recommended levels of 400 g per day GOP (2013).

Food safety is the key issue, especially in the developing countries. Production of the safe food is a big challenge and an important aspect of food quality assurance as well as public health. Generally, fruits and vegetables are irrigated with groundwater and other freshwater reservoirs. However, due to water scarcity in developing countries the reliance on groundwater has increased which is usually expensive and also poor in quality due to sodium adsorption, high electrical conductivity, sodium carbonate residues, and heavy metals Murtaza *et al*. (2008). Furthermore, domestic wastewater and industrial effluents have become an alternate source of irrigation, especially in the Peri-Urban areas. In

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developing countries, approximately 80% of the wastewater generated is estimated to be used to irrigate crops without prior treatment which is describe by Ensink *et al*. (2004). In Pakistan, approximately 30% of wastewater is used for irrigation, while rest of water is discharged into rivers without any treatment FAO (2002). The use of wastewater for irrigation purpose is controversial due to associated benefits and health concerns. Wastewater application has several disadvantages including the groundwater contamination, addition of heavy metals and organics in the soil resulting in a favourable environment for the growth of harmful micro-organisms Mapanda *et al*. (2007)

Dietary revelations to toxic metals (lead, cadmium and copper) and metalloid (arsenic) have been known as a danger to human health through the intake of different vegetables. This condition results in uneven degrees of health complications depending on the degree of exposures Demirezen and Aksoy (2006). Furthermore, the contaminated food intake with heavy metals can seriously decarease in immunological defences of malnutrition related disabilities, intrauterine growth retardation and a high incidence of upper gastrointestinal cancer (Zakir *et al*., 2009; Khan *et al*., 2008; Muchuweti *et al*., 2006). In addition, such metals like iron (Fe), zinc (Zn), copper (Cu) and manganese (Mn) require in small amount and known as trace elements. Among these, zinc and copper are essential for vital physiological and biochemical roles and necessary for sustaining health throughout the life. In developing countries, prompt and un-organized urbanization and industrialization have contributed to the high level of heavy metals in the urban environment (Wong *et al*., 2003). Heavy metals are persistent and non-biodegradable environmental pollutants which may be dumped on the surfaces of crop and later diffused into the tissues of the plants. In some areas, contamination of water by heavy metals is almost unavoidable because of the natural process (weathering of rocks) and human activities (domestic, agricultural and industrial effluents). Wastewater from the industries of electroplating, paint, mining or chemical laboratories frequently contains a high concentration of heavy metals including lead, cadmium and copper studies Raouf and Raheim (2016). These elements at above concentration not only could manage toxic effect in vegetables but also enter to the food chain, get bio-magnified and pose a significant threat to human health by Haiyan and Stuanes (2003). Application of wastewater for crop cultivation is known as an important source of heavy metals in soil Mapanda *et al*. (2005). Excessive level of metals in vegetables is specified due to use of untreated wastewater for a long time (Sharma *et al*., 2008). The presence of metallic compounds in fertilizers impart an extra source of metal contamination for vegetables (Yusuf *et al*., 2003). Atmospheric uptake of heavy metals from gas emission has also been known to contribute heavy metal pollution in vegetable crops Wiinikka *et al*. (2013).

The Pakistani population is increasingly growing leading to an immense demand for food to eat its needs. Most farmers are also illiterate and are unaware of the non-judicial use of agrochemicals. Pakistan is one of the leading country in which agrochemicals are widely used to increase their production. The frequent application of agrochemicals also contaminated the soil and water sources. Ultimatitly, these residues pave the way in the human body through oral, dermal and inhalation. The recent advances in the analytical chemistry have made it possible to even detect the traces of these toxic elements resulting in serious threat to the export of fruits and vegetables to the technically advanced countries in WTO scenario. This situation demands screening of locally produced vegetables for heavy metals as well as other toxins not only for export purpose but also for the safeguard of the human health. The aim of this study was to investigate the heavy metals concentration in highly consumed vegetables grown in Peri-Urban areas of two major divisions of Punjab and also evaluate their associated health risk among the local population. To the best of our knowledge, no or very few studies of this kind had been reported in the proposed study location with rarely focusing potential health risks of heavy metals exposure.

Materials and Methods

Procurement of raw material. Total of 51 vegetables samples *i.e.* potato (*Solanum tuberosum*), onion (*Allium cepa*), carrot (*Daucus carota*), turnip (*Brassica rapa*), cucumber (*Cucumis sativus*), tomato (*Solanum lycopersicum*), cauliflower (*Brassica oleracea*), pea (*Pisum sativum*), brinjal (*Solanum melongena*), bitter gourd (*Momordica charantia*), round gourd (*Praecitrullus fistulosus*), and okra (*Aesculantus malvaceae*) were collected from six Peri-Urban markets of two regions *i.e.* Multan and Rawalpindi, Punjab, Pakistan. The samples were packed into a polyethylene bag and immediately transferred to Food and Nutrition Laboratory of the National Institute of Food Science

and Technology for subsequent analysis. All reagents analytical grade were procured from Merck (Merck KGa A, Darmstadt, Germany).

Preparation of vegetable samples. The selected vegetables were rinsed with tap water in order to remove adhered soil, dust, dirt and other contaminants. The collected samples were reduced to an appropriate size by using hand knife. The reduced samples were kept in the dehydrator (Harvest Saver R-5A, commercial dehydrator systems Inc. Oregon, USA) at 55-60 ºC till complete dryness. The dried vegetable samples were ground into fine powder using a household blender (WF-8814 West Point, France) and then stored in polyethylene bags. This dried sample was further used for determination of heavy metals.

Sample analysis. The heavy metals (cadmium, chromium, lead, nickel, zinc, manganese and iron) in vegetable samples were determined by following the procedure described by Huang *et al*. (2013). Briefly 5g powdered sample was added into 100 mL Erlenmeyer flask. Concentrated HNO_3 (10 mL) and $HCLO_4$ (5 mL) were added to the sample followed by heating on a hot plate at 180 °C for 1.5 h till the volume was reduced to 1-2 mL and the samples become colorless. Finally, the sample was filtered into a clean volumetric flask and diluted with double deionized water. The solution was analyzed by mean of atomic absorption spectrometer (AA240, Varian Inc. Victoria, Australia) equipped with a graphite furnace for Ni, Cr, Cd, and Pb content.

Validation of analytical method. Blank sample and certified reference material (CRM) was prepared using the aforementioned digestion procedure and then run to check the accuracy of the analytical procedure. The reference material GBW10011 was purchased from National Research Center for Certified Material, China (NRCCRM). Qualitative results were obtained and the recoveries for all metals were ranged b/w 83.7-99.4%.

Survey on vegetables consumption. A survey was performed, while collecting the samples. A self-administered questioner was used to obtain information about vegetable consumption. Total 250 people both male and female with age limit of 18-50 years were invited to participate in this survey. Basic information age, body weight and daily consumption rate including species was gathered to evaluate the daily intake of metals and health risk assessment. Average body weight calculated was 63.5 Kg and the consumption rate of each vegetable is presented in (Table 3).

Calculation. Basic descriptive test (mean, standard deviation) was calculated by using SPSS for window version 18.0 (SPSS Inc., Chicago).

Estimated daily intake of heavy metals. The estimated daily intakes of heavy metals were calculated by using their mean value in vegetable sample. To evaluate EDIM following formula was used;

$$EDIM = FIR \times C_f \times Cm/Bw$$

Here, FIR represent food ingestion rate, C_f conversion factor (0.085) for conversion of fresh to dry weight, Cm represent heavy metal concentration and Bw mean average body weight.

Health risk index (HRI). Health risk index was calculated by using the formula proposed by USEPA (1998).

$$HRI = EDIM / RfD$$

R_fD represents oral reference dose for each toxic heavy metal. The R_fD for different heavy metals set by WHO/FAO are as follows; 0.007, 1.5, 0.025, 8.25, 0.3, 0.02, and 0.14 mg/Kg for Cd, Cr, Pb, Fe, Zn, Ni, and Mn, respectively . If the HRI > 1 then the exposed population likely to experienced significant health risk, if HRI < 1 then the population is considered as safe.

Results and Discussion

Metal concentrations in vegetables. Heavy metals have been widely known to adversely affect the nutritive value of agricultural goods on account of their lethal impact on a human being. Regulatory agencies such as Codex, EU, and FAO have identified the maximum residual levels (MRLs) of toxic metals and pesticides in human food in the WTO scenario. In Pakistan, the major apprehension is the non-judicial use of agrochemicals like fertilizers, pesticides and irrigation with sewage and industrial effluents especially in the Peri-Urban location of major cities. In the present study, the vegetables samples collected from Peri-Urban areas of Multan and Rawalpindi regions were analyzed for the determination of different heavy metals such as cadmium (Cd), chromium (Cr), lead (Pb), nickel (Ni), manganese (Mn), iron (Fe) and zinc (Zn) that compared with their maximum residual limits (MRLs). The mean values of the heavy metals in selected vegetables procured from Rawalpindi region are summarised in

(Table 1). Maximum and the minimum Cd contents in vegetable samples were found in pea (1.865 mg/Kg) and cauliflower (0.467 mg/Kg), respectively. Among Multan samples (Table 2) maximum Cd content was found in bitter gourd (0.786 mg/Kg) followed by okra (0.678), while the least content was observed in tomato (0.123 mg/Kg). Cd concentration in all vegetable samples exceeded the permissible limit (0.05 mg/Kg) as described by Joint FAO/WHO Expert Committee on Food Additive's Codex (2001). Rawalpindi samples were high in Cd content than Multan possibly because that site was close to the waste inclinator, which produces atmospheric pollution to the surrounding localities. Cadmium (Cd) being a serious accumulative body poison finds a way into the body through water, air, and food and cannot be removed by washing vegetables. Cadmium generally accumulates in liver and kidney Divrikli *et al*. (2003). In Pakistan, farmers are blindly using untreated wastewater for vegetable production especially in the Peri-Urban areas. High cadmium concentration in vegetables is attributed to the mechanism for its easy accumulation by plants Grant *et al*. (1998). In a previous study Naser *et al*. (2009) reported Cd concentration for tomato and cauliflower 3.83 and 3.67 mg/Kg, respectively. Likewise, in another study, Khan *et al*. (2013) reported the very high concentration of Cd in vegetables *i.e*. cauliflower and tomato grown in Peri-Urban areas of Lahore Pakistan. Maximum Cd concentration 6.7 mg/Kg was found in cauliflower followed by tomato 6.1 mg/Kg, respectively. Another study reported Cd contents in onion, cucumber and tomato 0.97, 0.64 and 0.41 mg/Kg, respectively Demirezen and Aksoy (2006).

In this study chromium (Cr) content in different vegetables ranged from (1.052-2.482 mg/Kg) with the highest level in onion and the lowest in pea (1.052), respectively (Table 1)**.** As shown in (Table 2) samples collected from Multan region showed a wide variation in the ranges of Cr content. The highest Cr content was found in cucumber (5.637 mg/Kg), while the lowest in round gourd (0.955 mg/Kg) (Table 2). Results revealed that Cr content was within the permissible limit (2.3 mg/Kg) among Rawalpindi samples, while Cr content in Multan samples was several folds higher than the permissible limit (2.3 mg/Kg) set by WHO/ FAO Codex (2001). This may be due to the difference in the texture of soil, pH, cation exchange capacity, soil organic contents or due to wastewater irrigation. In another study Shaheen *et al*., 2016 reported low content of chromium (0.2-1.11 mg/Kg) in different vegetable samples grown in Bangladesh. Our results were higher to those reported by Rehman *et al*. (2017) and lower than to those reported by (Khan *et al*., 2015; Liu *et al*., 2005).

In this study lead (Pb) content ranged from (3.04-5.64 mg/Kg) and (1.183-6.96 mg/Kg) among both regions (Table 1 and 2). The following decreasing order was found for Rawalpindi region pea > cauliflower > potato > turnip > cucumber > tomato > carrot. Among Multan

Table 1. Mean concentration (mg/Kg dw) and standard deviation of heavy metals in the vegetable samples collected from Rawalpindi region.

Vegetables	Heavy metal (mg/Kg)						
	Cd	Cr	Pb	Ni	Mn	Fe	Zn
Fruity(n=36)							
Cauliflower	0.4678±0.180	1.189±0.256	5.002±0.473	9.072±0.647	24.715±2.903	52.359±7.840	21.944±7.003
Tomato	1.0500±0.360	2.459±0.494	3.309±0.360	7.086±0.596	18.158±2.578	52.231±16.654	25.929±4.023
Cucumber	1.1422±0.110	1.282±0.514	4.245±0.755	9.106±0.379	16.311±3.390	73.941±28.149	28.418±3.831
Pea	1.8656±0.143	1.053±0.131	5.648±0.477	3.658±0.216	27.854±7.984	64.576±11.341	24.071±4.387
Rooty (n=36)							
Onion	0.9578±0.130	2.483±0.364	4.202±0.951	9.266±0.966	15.526±5.409	76.767±34.465	16.641±3.340
Potato	1.1111±.072	1.551±0.148	4.973±0.263	7.292±0.450	8.333±1.108	42.019±2.764	20.447±4.332
Turnip	1.3256±.087	1.634±0.149	4.266±0.862	5.286±0.282	9.642±1.478	37.512±3.390	25.831±5.165
Carrot	1.0967±.046	1.967±0.312	3.045±1.110	3.356±0.211	19.728±2.305	47.086±7.890	24.824±5.132
MRL							
FAO/WHO	0.05	2.3	0.1	10	-	465	60
SEPAC	0.2	0.5	-	10	-	-	100

MRL= Maximum Residual Limit ; dw = dry weight; n = number of samples; SEPAC = State environmental protection administration, China

region variation in lead content was in the following order; round gourd > potato > cucumber > bittergourd > onion > okra > tomato > cauliflower > brinjal. Lead content in all vegetable samples were above than the maximum permissible level (0.1 mg/Kg) as recommended by FAO/WHO Codex (2001). High concentration of lead (Pb) in these vegetables is attributed to pollutants in irrigation water and due to heavy traffic pollution. Parveen *et al*. (2003) also reported high concentration of lead in cucumber (1.72 mg/Kg) and tomatoes (1.56 mg/Kg), respectively and declare those vegetables unsafe for health. In another study Sharma *et al*. (2008) reported lead (Pb) concentration (1.56 µg/g) in cauliflower collected from different market sites of India. Present finding showed a higher concentration of lead (Pb) than those reported by (Zhou *et al*., 2007).

In vegetable samples, the maximum and the minimum value of the nickel (Ni) were 9.071 mg/Kg in cauliflower and 3.355 mg/Kg in carrot, respectively (Table 1). Nickel content in Multan sample was in the range of 4.07-7.90 mg/Kg (Table 2). Nickel content in all vegetable samples was below than the permissible limit of 10 mg/Kg set by joint FAO/WHO Codex (2001). In a previous study Naser *et al*. (2009) reported highest contents of nickel (Ni) in tomato ranged from (2.03-4.95µg/g) followed by cauliflower (1.69-4.44µg/g), respectively. Present result endorses the previous finding of Rehman *et al*. (2017) who reported nickel content in different vegetables ranging (1.83-7.69 mg/Kg). Present findings are also corresponding to the results of Yusuf *et al.* (2003).

The mean concentrations of manganese (Mn) ranged from (8.33-27.85 mg/Kg) in the Rawalpindi samples. Highest concentration was found in pea (27.85 mg/Kg) followed by cauliflower (24.69 mg/Kg), carrot (19.72 mg/Kg), tomato (18.15 mg/Kg), cucumber (16.31 mg/Kg), onion (15.52), and turnip (9.64 mg/Kg). The lowest concentrations were found in potatoes (8.33 mg/Kg). The highest and lowest concentrations were found in bitter gourd (45.56 mg/Kg) and brinjal (7.90 mg/Kg), An essential element is Manganese (Mn). Plant requires manganese to perform metabolic processes but their excessive accumulation can harm the consumer Sharma *et al*. (2006).

Iron (Fe) showed high concentration in vegetable samples. Among Rawalpindi samples, highest concentration was detected in onion (76.765 mg/Kg), while the least concentration was found in turnip 37.51 mg/Kg (Table 1). Vegetable samples from Multan showed higher concentration ranged from 46.758-156.784 mg/Kg (Table 2). Iron act as a micronutrients, if present in trace amount. Excess of iron associated with many health implications. In the literature, very high concentration of iron (364 µg/g) in different vegetables was reported by Ali and Al- Qahtani (2012). Similarly Khan and colleagues reported high iron

Table 2. Mean concentration (mg/Kg dw) and standard deviation of heavy metals in the vegetable samples collected from Multan region.

Vegetables	Heavy metal (mg/Kg)						
	Cd	Cr	Pb	Ni	Mn	Fe	Zn
Fruity(n=63)							
Cauliflower	0.1289±0.089	1.2088±0.476	1.8422±0.507	4.076±1.006	12.725±1.350	46.758±6.089	27.653±2.750
Tomato	0.1233±0.049	3.0533±0.369	1.9589±0.169	7.014±0.305	12.299±1.415	59.579±2.451	16.543±6.118
Cucumber	0.1356±0.077	5.6378±0.602	5.8422±1.356	6.984±2.387	31.036±21.078	91.253±24.010	28.535±3.811
Brinjal	0.3611±0.106	1.9556±0.417	1.1833±0.315	7.9045±.101	7.9045±.101	78.708±18.155	20.569±2.252
Round Gourd	0.3322±0.225	0.9556±0.201	6.9678±0.5271	6.0057±0.319	17.796±1.771	77.377±20.02	28.074±6.444
Bitter Gourd	0.7867±0.126	3.78±0.513	4.6189±0.431	7.7412±0.416	45.566±6.178	153.784±14.52	24.212±7.556
Okra	0.6789±0.087	1.6878±0.272	2.7911±0.303	4.1856±0.127	32.296±3.3094	121.298±7.211	23.797±5.706
Rooty(n=18)							
Onion	0.1267±0.04	1.9411±0.340	4.1656±0.411	7.2767±0.589	26.026±7.586	135.445±7.859	19.5±2.963
Potato	0.2356±0.067	2.7956±0.370	6.5211±0.464	4.2122±0.320	14.136±5.557	58.642±9.142	22.585±4.400
MRL							
FAO/WHO	0.05	2.3	0.1	10	-	465	60
SEPAC	0.2	0.5	-	10	-	-	100

MRL= Maximum Residual Limit ; dw = dry weight; n = number of samples; SEPAC = State Environmental protection Administration, China

Table 3. Estimated daily intake of metals (EDIM) through consumption of different vegetables grown in both regions.

Vegetables	Consumption g/day/person	Estimated daily intake of metals (mg/day)						
		Cd	Cr	Pb	Ni	Mn	Fe	Zn
Cauliflower(R)	120	7.514E-05	0.000191	0.000804	0.001458	0.00397	0.008412	0.0035248
Cauliflower(M)	120	2.071E-05	0.000195	0.000296	0.000655	0.002044	0.007511	0.0044418
Tomato (R)	130	0.0001827	0.000428	0.000576	0.001234	0.001859	0.009089	0.004512
Tomato(M)	130	2.146E-05	0.000532	0.000341	0.001221	0.001259	0.010368	0.0028786
Cucumber (R)	74	0.0001	0.000126	0.000421	0.000902	0.001616	0.007325	0.0028149
Cucumber (M)	74	0.0000	0.000559	0.000579	0.000692	0.003074	0.009040	0.0028265
Pea	120	0.0003	0.000170	0.000908	0.000588	0.004474	0.010373	0.0038665
Brinjil	110	0.0001	0.000289	0.000175	0.001168	0.001167	0.011621	0.0030369
Round gourd	118	5.248E-05	0.000151	0.001101	0.000949	0.002811	0.012222	0.0044343
Bitter gourd	104	0.0001096	0.000527	0.000644	0.001078	0.006343	0.021409	0.0033705
Okra	170	0.0001545	0.000385	0.000636	0.000953	0.007349	0.027603	0.0054152
Onion (R)	90	0.0001154	0.000300	0.000507	0.001117	0.00187	0.009249	0.0020048
Onion (M)	90	1.527E-05	0.000234	0.000502	0.000877	0.003135	0.016318	0.0023492
Potato (R)	86	0.0001280	0.000179	0.000751	0.000840	0.00097	0.004838	0.0023538
Potato (M)	86	2.713E-05	0.000322	0.000751	0.000485	0.001646	0.006751	0.0025999
Turnip	86	0.0001527	0.000189	0.000492	0.000609	0.00111	0.004319	0.0029736
Carrot	128	0.0001880	0.000337	0.000522	0.000575	0.00338	0.008068	0.0042534
EDIM from all vegetables		0.0017240	0.005107	0.009998	0.015393	0.0184507	0.184507	0.0576566
MTDI0.021			0.2	0.21	0.3	2-5	-	60

M = sample collected from Multan; R = Sample collected from Rawalpindi; MTDI = Maximum tolerable daily intake

contents (73-190 mg/Kg) in cauliflower and tomato, which is similar to the current finding of Khan *et al*. (2013). Farmer are not well trained in Pakistan and blindly use wastewater to irrigate vegetables and other crops without taking into account their consequences. Heavy metals accumulations is due to direct use of untreated wastewater. Furthermore, non-judicial applications, of agricultural practices imparts to food stuffs an alternative source of heavy metal.

Zinc (Zn) concentration in vegetable samples varied in the range of 16.64-28.41 mg/Kg with least concentration in onion and maximum concentration in cucumber (Table 1). Among Multan samples, highest concentration was observed in cucumber (28.53 mg/Kg), whereas lowest concentration was detected in tomato (16.54 mg/Kg) (Table 2). All the vegetable samples were within the maximum permissible limit of 60 mg/Kg set by FAO/WHO. Zinc is most important and a vital trace component for higher plants and animals and also play a role in energy metabolism, transcription and translation due to the variety of enzyme systems (Meunier *et al*., 2005). In some soil, the higher amount of zinc is associated to human activities and it is potentially dangerous. Excessive contents in soil results in phytotoxicity and eventually enter to the food chain. Results obtained from the present study were similar to those obtained by Mohammad *et al*. (2003) who found Zn contents in cucumber 32.3µg/g and 20.08 mg/Kg, respectively.

Estimated daily intake of heavy metals. Heavy metals are comon componeuts in the Earth's crust that are not degradable. There are several pathways of heavy metal exposure in our body. These enter our body through different routes like food, drinking water, and air and cause many serious effects even at very low concentration. The explantion behind the toxicity of heavy metals is that they are noncompetitive inhibitors for numerous enzymes Esposito *et al*. (2001). Table 3 summarizes the estimated daily intake of metals (EDIM) of seven metals (Cd, Cr, Pb, Ni, Mn, Fe, and Zn along with maximum tolerable daily intake (MTDI). EDIM were evaluated following the mean concentrations of each metal in each vegetable and the respective consumption rate among the masses. Iron (Fe) showed highest daily intake 0.185 followed by Zn (0.058), Mn (0.049), Ni (0.015), Pb (0.009), Cr (0.0051), and Cd (0.0018) mg/day, respectively. It is obvious from the finding that the daily intake of all the metals was less than the respective MTDI.

Table 4. Health risk assessment of heavy metals from consuming different vegetables grown in both regions.

Vegetables	Health risk index						
	Cd	Cr	Pb	Ni	Zn	Fe	Mn
Cauliflower(R)	1.074E-05	1.274E-07	3.214E-05	7.286E-05	1.175E-05	1.019E-06	2.835E-05
Cauliflower (M)	2.958E-06	1.295E-07	1.184E-05	3.273E-05	1.481E-05	9.104E-07	1.459E-05
Tomato (R)	2.611E-05	2.853E-07	2.304E-05	6.166E-05	1.5041E-05	1.102E-06	1.327E-05
Tomato(M)	3.066E-06	3.543E-07	1.364E-05	6.103E-05	9.596E-06	1.257E-06	8.992E-06
Cucumber (R)	1.643E-05	8.460E-08	1.682E-05	4.510E-05	9.384E-06	8.878E-07	1.153E-05
Cucumber (M)	1.919E-06	3.724E-07	2.315E-05	3.9E-05	9.422E-06	1.096E-06	2.195E-05
Pea	4.282E-05	1.127E-07	3.629E-05	2.938E-05	1.289E-05	1.258E-06	3.195E-05
Brinjil	7.596E-06	1.925E-07	6.989E-06	5.836E-05	1.013E-05	1.409E-06	8.336E-06
Round gourd	7.496E-06	1.007E-07	4.403E-05	4.744E-05	1.479E-05	1.482E-06	2.007E-05
Bitter gourd	1.565E-05	3.509E-07	2.573E-05	5.389E-05	1.124E-05	2.595E-06	4.530E-05
Okra	2.207E-05	2.561E-07	2.545E-05	4.763E-05	1.806E-05	3.346E-06	5.249E-05
Onion (R)	1.649E-05	1.994E-07	2.025E-05	5.582E-05	6.683E-06	1.121E-06	1.336E-05
Onion (M)	2.181E-06	1.559E-07	2.008E-05	4.389E-05	7.831E-06	1.978E-06	2.239E-05
Potato (R)	1.828E-05	1.190E-07	3.003E-05	4.197E-05	7.846E-06	5.864E-07	6.931E-06
Potato (M)	3.875E-06	2.146E-07	3.003E-05	2.425E-05	8.667E-06	8.183E-07	1.176E-05
Turnip	2.181E-05	1.254E-07	1.965E-05	3.043E-05	9.913E-06	5.235E-07	7.927E-06
Carrot	2.685E-05	2.247E-07	2.087E-05	2.873E-05	1.418E-05	9.779E-07	2.414E-05
THRI	0.000247	3.405E-06	0.000399	0.000770	0.000193	2.237E-05	0.000344

M = sample collected from Multan; R = Sample collected from Rawalpindi; THRI = Total health risk index

Assessment of health risk index (HRI). Health risks from the mass consumption of contaminated vegetables is evaluated and summarised in the (Table 4). The health risk index < 1 considered as safe Zheng *et al*. (2007). Results showed that HRI of all the metals fell within the permissible limit (< 1) which indicates that exposed population due to the consumption of these vegetables. The following decreasing order was observed for heavy metals in all vegetable samples NI > Pb > Mn > Cd > Zn > Cr > Fe, respectively.

Conclusion

This study revealed the presence of heavy metals in selected vegetables grown in Peri-Urban areas of Punjab as well as estimated daily intake and the associated health risk by consuming these vegetables. Among both regions, a wide range of variation for different heavy metals in different vegetables were observed. Results revealed that cadmium (Cd), and lead (Pb) content was exceeding their maximum residual limits (MRLs) set by FAO/WHO. From the consumption perspective, EDIM and HRI of all metals were below than the permissible limit hence it is concluded that consumption of these vegetables poses no significant threat to the local population.

Acknowledgement

I would like to thanks Dr. Khalid Bashir Principle investigator of project Food Utilization Among Poor Households in Peri-Urban Areas of North and South Punjab Pakistan sponsored by IFPRI for the provision of chemicals and standards.

Conflict of Interest. The authors declare no conflict of interest.

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Pak. j. sci. ind. res. Ser. B: biol. sci. 2021 **64B**(1) 64-74

Effect of Salinity on Emergence and Early Growth Stages of Aromatic and Non-Aromatic Rice (*Oryza sativa* L.) Genotypes

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(received July 31, 2018; revised January 13, 2019; accepted January 17, 2019)

Abstract. Salinity hampers the production of many field crops in the region including rice (*Oryza sativa* L.), while commonly classified as salt sensitive within the same species, the level of sensitivity varies between genotypes. This study investigated the salinity tolerance of 28 rice genotypes, including 9 aromatics and 19 non-aromatics. Sixty seeds of each genotype were initially sown in trays (24×18 inches) during the 1st week of June, by of using four salt treatments (0, 40, 80 and 120 mM NaCl+$CaCl_2$ @ 20:1). The experiment was laid down in a completely randomized design with four replicates in laboratory conditions, at Shah Abdul Latif University Khairpur, Sindh, for the period of twenty-five days. A significant reduction in agro-morphological parameters was observed against all salinity levels. Based on reduction in dry matter yield, all rice genotypes were found tolerant at 40 mM. Eleven rice genotypes were found tolerant, fourteen were moderately tolerant, one was moderately sensitive and remaining two genotypes were found sensitive at 80 mM salinity level. Furthermore, none of the genotypes were able to with stand 120 mM of salinity. The genotypes Khushboo, DR-83 and Mahek performed meager and showed more than 50% reduction over control and categorised as sensitive, with the genotypes Latifee, DR-67 and DR-92, DR-51 and IR-6 are categorised as tolerant with a reduction of less than 20% over control based on dry matter yield reduction against all salinity treatments at the early seedling stage. However, these genotypes cannot be justified as tolerant only on the basis of their improved performance at early growth stage. Hence, these genotypes are suggested to be studied further at other advanced growth stages up to maturity to evaluate their response under a saline environment.

Keywords: salinity tolerance, aromatic and non-aromatic rice, genotypes, germination, survival percentage, dry matter yield

Introduction

Rice, a major export commodity, for export, play a key role in Pakistan agrarian based economy. The country earned 26.5 million US$ during 2017 through its export (PBS, 2018). It is the third largest crop in terms of area, cultivating over 9,050,000 hectares and second principal food grain crop of Pakistan, produced 26,500,000 tonnes during 2017-18. Punjab and Sindh are the major rice producing provinces of Pakistan, contributing approximately 90% of the overall rice production (PBS, 2018). Rice is one of the nutritionally imperious grain crops feeding more than three billion people around the world (Ghosh *et al*., 2016; Mohammadi-Nejad *et al*., 2010). A 100 g of rice provide 345 Kcal energy, 78.2 g of carbohydrates, 6.8 g of protein and a significant amount of suggested Zinc and Niacin (Ali *et al*., 2014; Gopalan *et al*., 2007). Rice varieties with some special traits such as fragrance, better taste and higher cooking quality also provide extra value in socio-economic aspects.

Salinity is one of the ecological challenges after drought, reducing crop production over 800 million hectares throughout the world (Islam *et al*., 2013). Generally, the Na^+ and Cl^- ions resulting from NaCl, are the major cause of salinity. A high concentration of such ions may affect the functioning of plant cell, resulting in reduced growth and lessen yield (Läuchli and Grattan, 2007). High salinity in the flooded soil is one of the main factors that depress water availability to root cells of rice plants due to reduced osmotic potential, consequently, inhibiting growth, development and grain yield of rice (Ganie *et al*., 2016; Molla *et al.*, 2015). Salinity causes oxidative stress and disparity in nutrition, due

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to high concentration of specific ions unbalancing osmotic regulation (Ashraf, 2010; Noreen *et al*., 2010). This further inhabits germination, affects the growth of seedlings, and crush leaf enlargement, subsequently providing small leaf blade for photosynthetic activities and less dry matter of plant (Ahmad *et al.*, 2010; Ashraf, 2010).

During the early seedling (2-3 leaf) stage, rice remains very sensitive studied by (Mondal and Borromeo, 2016). Salinity reduces seedling survival and results in low crop standing (Pushpam and Rangasamy, 2000). Salinity reduces the weight of the shoot, the number of leaves, shoot and root length and the surface area of the roots (Meloni *et al*., 2001). Some high concentrations of Na^+ and/or Cl^- in plants cause wilting of leaf tips and necrosis (Zafar *et al*., 2015). Salinity stressed suppress development can be distinguished by measuring effects immediately upon salt exposure or after several days to weeks (Roy *et al*., 2014). Soil salinity is one of many factors that poses a major challenge to sustainable agriculture in Pakistan (Hussain *et al*., 2012). Most of the saline soils have originated from various natural processes, such as rocks weathering movement of salt traces through irrigation water (Munns and Tester, 2008).

In Asia, salinity has affected more than twenty million hectares of arable land and loss about half of the predictable fertile land (Huyen *et al*., 2013; Nazar *et al*., 2011). Moreover, the human population around the globe is increasing rapidly. Either cropping area or production is to be increased to feed this growing population of the world. The supply of agricultural land availability is slowly declining with speed as population grows, as much of the land is being converted into residential and commercial areas for community. On the other hand, agriculture is suffering from serious damage of biotic and abiotic stresses. Scientists around the globe are putting their best efforts to improve genotypes to combat stress affected environments and meet the challenges of the present era (Sankar *et al*., 2011). The screening of salt tolerant species is a reliable method to cope the salinity and produce a better production (Shannon *et al*., 1998). Therefore, this study was conducted to screen out the salt-tolerant rice genotypes at early seedling stages and to test their salt tolerance levels in saline environment.

Materials and Methods

In the 1st week of June 2016, the experiment was conducted at Department of Botany, Shah Abdul University Khairpur, Sindh, Pakistan. Twenty eight rice genotypes were selected, including 09 aromatic (Mahek, Khushboo, DR-61, DR-62, DR-63, DR-66, DR-67, Super Basmati and Lateefi), and 19 non-aromatic (Kanwal-95, Shahkar, Sarshar, Sada Hayat, Shadab, Shandar, NIA-19-A, NIA-625, DR-50, DR-51, DR-52, DR-57, DR-59, DR-82, DR-83, DR-92, IR-6, IR-8 and Shua-92 was used as salt-tolerant check variety) for analysis. The seeds of genotypes were sourced from RRI Dokri, Sindh, Pakistan and NIA Tandojam, Sindh, Pakistan. The seeds were sterilized for 30 min on the surface with 1% industrial bleach and washed four times with distilled water. After rinsing, sixty seeds of each genotype were sown in 10 Kg air dried soil filled trays (24×18 inches). The soil was analyzed before experiments (Table 1). The trays were arranged in completely randomized design (CRD) with four repeats and four salt treatments (0, 40, 80 and 120 mM, NaCl+$CaCl_2$ @ 20:1) were applied. The salt solution was prepared by following method. For the conversion of molar into mM following formula was used:

$$\text{mM} = \frac{\text{Molar weight of NaCl}}{1000} \times \text{salt treatments}$$

The resulting weight of salt is added in distilled water per liter to make a salt solution. The temperature of the laboratory was maintained at 28 °C.

Emergence percentage was measured at interval of one week of sowing. Twenty five days old seedlings were harvested and washed with distilled water. Survival percentage, shoot height and root length (cm), shoot and root dry weight (g), shoot/root ratio and dry matter yield (g) were recorded at the time of seedlings harvest**.** Seedlings were wrapped in paper bag and kept in an

Table 1. Physico-chemical properties of soil used in experiment

Soil properties	Value
Texture	
Sand	25.2%
Silt	41.3%
Clay	33.5%
Textural class	Clay loam
Electrical conductivity (1:5)	2.2 d/Sm
pH (1:5)	7.4
Organic matter	0.75%
$CaCO_3$	6.0%

oven at 65 °C for 24 h to a constant dry weight. The germination percentage (GP) and survival percentage (SP) was calculated using the formula suggested by Raun *et al*. (2002). Whereas, percent reduction over control (PROC) was computed using the formula suggested by Ali *et al*. (2014), as following:

$$GP = \frac{\text{Total number of germinated seeds}}{\text{Total number of germinated seedling}} \times 100$$

$$SP = \frac{\text{Total number of survival seedling}}{\text{Total number of germinated seedling}} \times 100$$

$$PROC = \frac{\text{Volume in control - Value in saline environment}}{\text{Value in control}} \times 100$$

Genotypes were categorised as tolerant (T) having 0-20% reduction, moderately tolerant (MT) and moderately sensitive (MS), having 21-40% and 41-60% reduction respectively while, sensitive (S) more than 60% reduction of their aggregate dry material (shoot and root) at various levels of salinity stress (Ologundudu *et al*., 2014; Hakim *et al*., 2010). The data were analyzed by performing two-way ANOVA ($P<0.05$) and means were compared by least significant difference (LSD) using statistical software namely "Statistix version 8.1".

Results and Discussion

Germination percentage (GP). The germination of all aromatic and non-aromatic rice genotypes significantly decreased when exposed to salinity treatments (Table 2). A negligible effect of salinity was observed at 40 mM salinity with least (14.6) average PROC of all genotypes (Fig.1a). However, genotype DR-63 showed maximum (37.77) PROC even at this level of salinity. The most severe effect of salinity was observed at 120 mM salt concentration, where maximum (47.86) average PROC of all genotypes was recorded. The genotypes DR-92, DR-51 and Latifee performed better, both showed least (≤ 16.51) average PROC at all salinity levels, whereas genotypes DR-63, DR-61 and Sada Hayat germinated poorly in saline environment and showed maximum (>50) average PROC as compared to other genotypes. Generally, PROC increases with the rise in salinity level (Fig.1a).

Survival percentage (SP). Increased salinity levels significantly decreased the survival percentage of all rice genotypes (Table 2) as compared to control salinity treatment. Salinity level 120 mM showed most harmful effects on seedling survival, where more than 60% reduction was observed as compared to control (Fig. 1b). Genotypes DR-83 and Khushboo could not with stand the hazardous effect of salinity and completely died at 80 mM salinity. Genotypes DR-92, DR-51 and IR-6 remained successful with least average (≤ 21) PROC.

Shoot height and root length. Shoot height of all aromatic and non-aromatic rice genotypes was reduced when exposed to salt stress (Table 3). Mild effect of salinity was observed at 40 mM salinity, where on an average less than ten PROC was recorded. The most significant effect of salinity was noted at 120 mM salinity treatment, where on an average 56.62 PROC was observed. The maximum (68.98, 68.73 and 41.67) average PROC was observed in genotypes Khusboo, DR-83 and DR-57 respectively, whereas minimum average (<20) PROC was recoded in genotypes Latifee, DR-92 and DR-51 (Fig.1c). Similarly, root length was also decreased with increased levels of salinity (Table 4). The minimum (11.24) average PROC in root length was observed at 40 mM salinity which raised to maximum (61.91) average PROC at maximum (120 mM) salinity level. The genotypes DR-92, NIA-19A and Super Basmati showed minimum (≤ 26) average PROC in root length at all salinity levels, whereas maximum (69.07, 68.49 and 50.37) average PROC for same trait at all salinity treatments was recorded in genotypes Khushboo, DR-83 and DR-52, respectively (Fig.1d).

Shoot and root dry weight. Shoot dry weight of all rice genotypes was significantly reduced with higher salinity levels as compared to control (Table 3). All genotypes performed well with a least PROC (<9%) at 40 mM salinity treatment (Fig.1e). DR-92, Latifee and DR-51 showed better response with minimum (18.82, 22.64 and 22.75) average PROC, whereas Khushboo, DR-83 and Mahek performed meager and showed maximum average PROC at all salinity levels (Fig.1e). Similarly, root dry weight of all genotypes was also decreased with increased salinity levels (Table 4), on average, at 40, 80 and 120 mM salinity, root dry weight of all genotypes reduced (8.24, 24.54 and 51.48%) respectively as compared to control.

The lowest (14.18, 16.99 and 17.39%) reduction on average at all salinity levels were observed in genotypes DR-67, DR-92 and DR-51 respectively, whereas highest (68.69, 67.50 and 50.45%) average reduction at all

salinity levels in root dry weight were recorded in genotypes Khushboo, DR-83 and Mahek respectively as compared to control (Fig.1f).

Shoot/root ratio. In all genotypes, shoot/root ratio was significantly decreased with increased salinity levels (Table 5). The highest (2.51, 2.44 and 2.42) shoot/root ratio at all salinity levels were observed in genotypes DR-92, DR-51 and Latifee respectively, whereas lowest (0.94, 1.27 and 1.28) shoot/root ratios were recorded in genotypes DR-83, IR-8 and DR-52, respectively. The more average PROC (>60) was observed in genotypes Khushboo, DR-83 and Shandar respectively, while mild average PROC (≤ 13.5) was recorded in genotypes DR-92, DR-51 and IR-6 (Fig.1g).

Classification of genotypes. All genotypes were classified as tolerant (T), medium tolerant (MT), medium sensitive (MS), and sensitive (S) on the basis of dry matter production at different salinity levels (Table 5). All rice genotypes fall in the category of T at 40 mM. Eleven rice genotypes fall in the category of T, fourteen in the category of MT, one in the category of MS and remaining two genotypes fall in the category of S at 80 mM salinity level. No any rice genotype could qualify for T category at 120 mM salinity.

Table 2. Effect of salinity on germination and survival percentages of aromatic and non-aromatic rice genotypes grown in solution culture. The number shows the mean germination and survival under various salinity treatments. ($NaCl + CaCl_2$ @ 20:1 salt concentrations (mM))

Genotypes	Germination percentage (GP)					Survival percentage (SP)				
	T1	T2	T3	T4	Genotype mean	T1	T2	T3	T4	Genotype mean
Sarshar	85.0	75.4	70.5	53.0	71.0	94.1	93.0	76.9	34.3	74.6
Shadab	82.3	75.0	55.0	30.0	60.6	94.0	84.4	69.0	34.4	70.5
Shandar	86.6	78.4	71.3	48.0	71.1	89.8	85.7	51.1	34.1	65.2
Shua-92	87.2	81.4	75.2	70.0	78.5	96.3	93.2	83.2	67.5	85.0
NIA-19A	86.0	76.6	70.1	65.0	74.4	94.4	89.1	73.1	54.0	77.7
NIA-625	86.6	70.0	50.4	30.0	59.3	85.0	71.3	56.6	27.5	60.1
DR-50	85.0	70.5	50.3	35.0	60.2	95.3	76.1	72.9	20.6	66.2
DR-57	86.0	73.4	70.0	46.1	68.9	88.4	73.5	55.9	0.0	54.4
DR-83	85.0	68.3	40.0	30.0	55.8	90.8	76.4	0.0	0.0	41.8
DR-52	86.0	75.0	70.1	44.8	69.0	88.0	80.0	69.4	0.0	59.3
DR-51	87.0	83.5	76.4	60.3	76.8	94.1	91.2	74.3	57.6	79.3
DR-82	85.0	66.2	45.0	30.1	56.6	84.7	76.6	64.2	30.3	63.9
DR-92	88.1	81.6	73.0	71.1	78.4	94.4	91.4	68.4	66.7	80.2
IR-6	87.0	76.1	73.3	68.0	76.1	94.3	90.5	82.1	50.0	79.2
IR-8	85.0	76.0	50.5	30.0	60.4	88.6	71.2	65.6	28.5	63.5
Sada Hayat	85.0	61.6	38.0	24.8	52.4	86.3	75.0	62.9	29.0	63.3
Kanwal-95	85.0	70.0	39.9	34.6	57.4	90.6	75.0	66.1	29.0	65.2
Shahkar	86.4	73.1	70.0	64.6	73.5	94.0	80.8	76.1	52.2	75.8
DR-59	82.0	70.4	59.6	45.0	64.2	88.2	75.0	62.2	35.0	65.1
Mahek	78.0	71.6	68.3	24.8	60.7	87.0	77.0	64.9	0.0	57.2
Khushboo	81.2	65.0	45.0	25.0	54.1	87.0	77.1	0.0	0.0	41.0
DR-62	84.0	74.5	60.1	51.3	67.5	87.0	78.2	67.6	30.0	65.7
DR-66	94.0	85.1	81.5	50.0	77.7	94.0	85.1	81.5	50.0	77.7
DR-67	85.0	76.8	74.6	60.1	74.1	94.0	79.2	76.1	62.0	77.8
DR-63	80.4	50.0	35.1	28.0	48.4	95.3	72.7	52.2	28.0	62.1
Super Basmati	86.0	81.0	70.0	36.5	68.4	94.3	88.2	74.3	52.6	77.4
DR-61	76.2	50.0	35.3	25.2	46.7	94.5	72.7	54.8	37.4	64.8
Latifee	85.0	76.2	71.7	65.0	74.5	94.2	88.0	73.0	60.0	78.8
Treatment mean	84.85	72.59	60.	44.5		91.4	81.0	63.4	34.7	
	Genotype (G)		**Salinity**	**G X S**		**Genotype (G)**		**Salinity**	**G X S**	
S.E.D	0.62		0.23	1.25		0.48		0.18	0.96	
L.S.D (0.05%)	1.23***		0.46***	2.47***		0.94***		0.35	1.89***	

Although eight genotypes were found successful for MT, eleven for MS and remaining nine were categorised as S at 120 mM salinity.

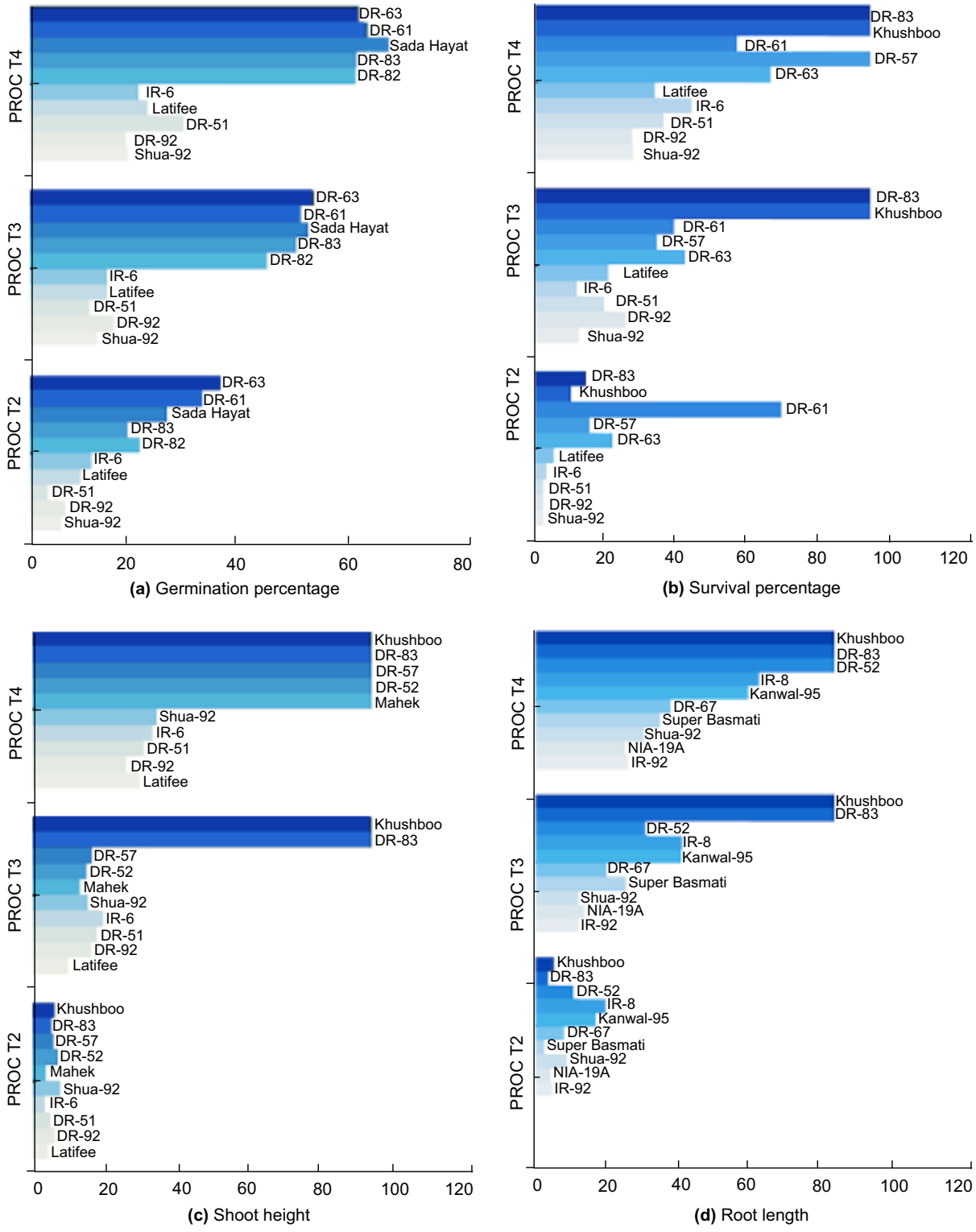
In Pakistan, agriculture is facing plenty of problems and issues regarding crop production (Muzaffar *et al*., 2015; Rao *et al*., 2013). Several approaches are being attempted to cope against those problems and issues (Awan *et al*., 2015; Nasir *et al*., 2014). Soil salinity, an abiotic stress is also one of the major problems which not only reduce the crop growing area but also its yield throughout the world (Kronzucker *et al*., 2008). Rice is susceptible to salt-stress, especially during the period of seedling growth (Zafar *et al*., 2015). It is therefore, vital to screen out the best genotypes that produce better yield in saline conditions (Zeng and Shannon, 2002).

In present study, the influence of salinity remained adverse on emergence, survival and early growth of aromatic and non-aromatic rice genotypes. Results showed a significant reduction in almost all agronomic parameters. However, the rate of reduction varied in both aromatic and non-aromatic rice genotypes. The symptoms of salt injury could be visualized physically.

Table 3. Effect of salinity on shoot height and shoot dry weight of aromatic and non-aromatic rice genotypes grown in solution culture. The number shows the mean shoot height (cm) and shoot dry weight (g) under various salinity treatments. ($NaCl + CaCl_2$ @ 20:1 salt concentrations (mM))

Genotypes	Shoot height (cm)					Shoot dry weight (g)				
	T1	T2	T3	T4	Genotype mean	T1	T2	T3	T4	Genotype mean
Sarshar	15.1	13.4	11.9	5.9	11.6	0.089	0.08	0.066	0.027	0.066
Shadab	16.0	12.6	11.1	7.9	11.9	0.097	0.082	0.068	0.023	0.068
Shandar	15.5	12.6	11.4	9.0	12.1	0.088	0.076	0.067	0.033	0.066
Shua-92	16.2	14.8	13.5	10.2	13.7	0.131	0.123	0.111	0.061	0.107
NIA-19A	16.7	15.4	12.5	10.7	13.8	0.089	0.081	0.074	0.049	0.073
NIA-625	15.7	13.7	12.9	8.4	12.7	0.084	0.078	0.06	0.027	0.062
DR-50	15.7	14.5	12.7	7.0	12.5	0.076	0.072	0.058	0.028	0.059
DR-57	14.9	13.9	12.2	0.0	10.3	0.081	0.077	0.063	0	0.055
DR-83	13.2	12.4	0.0	0.0	6.4	0.074	0.071	0	0	0.036
DR-52	14.9	13.7	12.4	0.0	10.3	0.083	0.073	0.058	0	0.054
DR-51	15.7	14.8	12.7	10.5	13.4	0.126	0.115	0.102	0.075	0.105
DR-82	16.2	15.0	12.4	9.6	13.3	0.081	0.07	0.06	0.045	0.064
DR-92	16.8	15.6	13.8	12.1	14.6	0.124	0.121	0.102	0.079	0.107
IR-6	16.1	15.5	12.7	10.4	13.7	0.116	0.106	0.081	0.07	0.093
IR-8	17.8	14.7	13.1	7.1	13.2	0.077	0.068	0.06	0.035	0.06
Sada Hayat	16.2	14.5	12.4	7.9	12.8	0.082	0.078	0.06	0.041	0.065
Kanwal-95	19.0	15.2	14.8	9.9	14.7	0.077	0.065	0.058	0.045	0.061
Shahkar	15.8	14.7	14.1	8.7	13.3	0.117	0.109	0.081	0.07	0.094
DR-59	14.6	13.3	12.3	7.6	11.9	0.076	0.07	0.057	0.037	0.06
Mahek	14.7	14.0	12.5	0.0	10.3	0.106	0.099	0.047	0	0.063
Khushboo	14.4	13.4	0.0	0.0	7.0	0.101	0.091	0	0	0.048
DR-62	17.1	16.3	13.9	5.4	13.2	0.084	0.076	0.062	0.03	0.063
DR-66	17.7	16.3	14.3	6.4	13.7	0.081	0.072	0.066	0.038	0.064
DR-67	23.1	20.5	16.0	13.5	18.3	0.108	0.095	0.08	0.07	0.088
DR-63	17.2	13.2	12.3	8.3	12.8	0.079	0.074	0.062	0.025	0.06
Super Basmati	18.1	16.5	12.9	9.5	14.3	0.096	0.086	0.075	0.055	0.078
DR-61	16.5	15.1	12.5	6.4	12.6	0.081	0.078	0.06	0.038	0.064
Latifee	15.7	14.9	14.0	10.7	13.8	0.106	0.102	0.085	0.059	0.088
Treatment mean	16.3	14.7	12.04	7.24		0.093	0.085	0.065	0.038	
		Genotype (G)		**Salinity**	**G X S**		**Genotype (G)**		**Salinity (S)**	**G X S**
S.E.D		0.42		0.15	0.84		1.90		7.20	3.81
L.S.D (0.05%)		0.82***		0.31***	0.65***		3.75***		14.42***	7.51***

Burning of older leaf tips were noticed at initial stage which led to completely dying of leaf. Germination of all rice genotypes including aromatics and non-aromatics were affected by salinity. Reduction in their germination percentage increased with the increasing intensity of salinity. This may have resulted from the imbalance of osmotic pressure caused by the concentration of salts (Anbumalarmathi and Mehta, 2013). Other studies



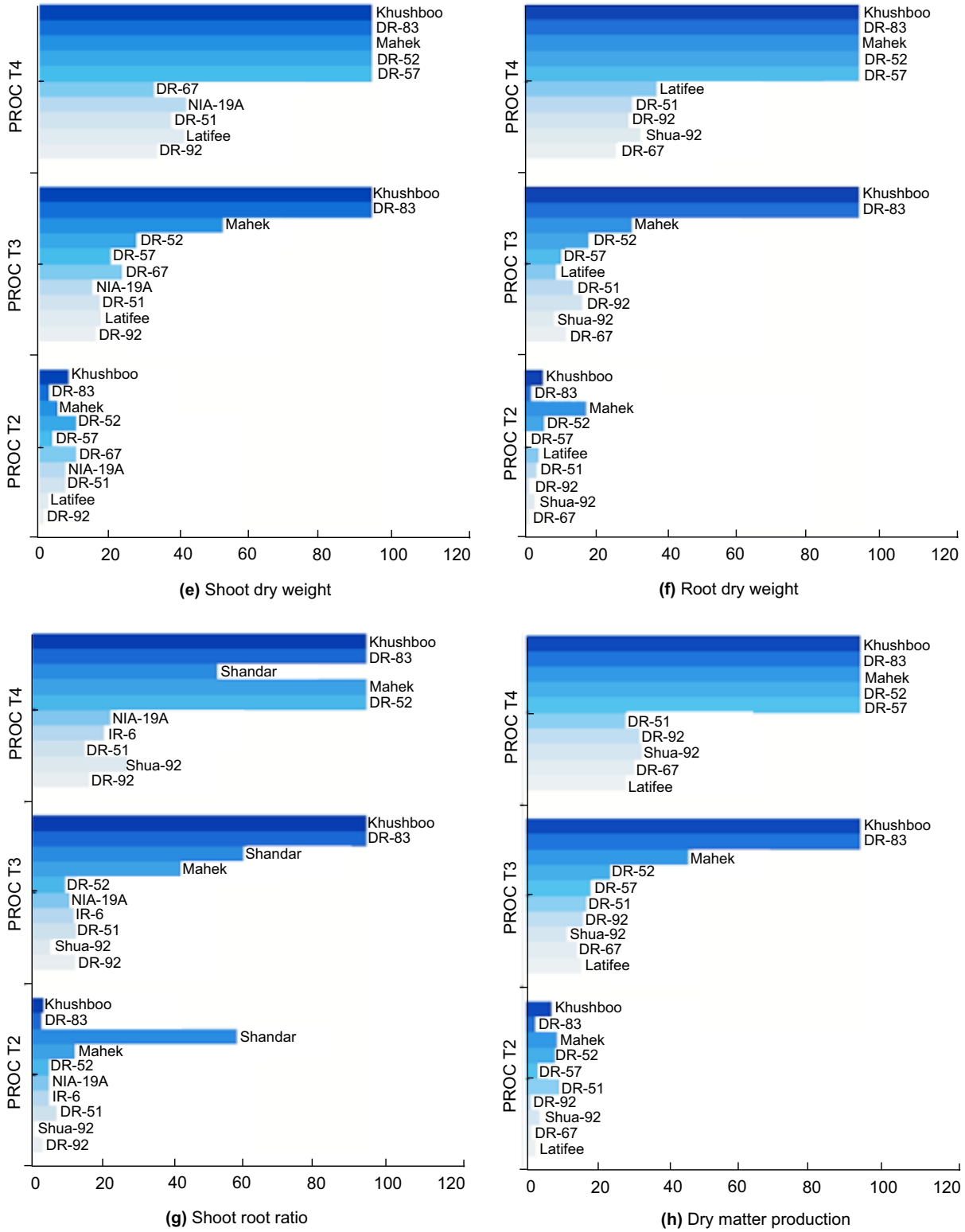


Fig. 1. Reduction (PROC) in the agro-morphological parameters of rice genotypes at three salinity treatment. (a) Germination percentage, (b) Survival percentage, (c) Shoot height, (d) Root length, (e) Shoot weight, (f) Root dry weight, (g) Shoot root ratio and (h) Dry matter production. Only ten genotypes having the highest and lowest impact of salinity are plotted in the figure for the clarity.

(Hakim *et al*., 2010; Zafar *et al*., 2015) have also reported rice genotypes susceptible for germination percentage against salinity stress particularly in salt sensitive varieties. Germination percentage is among many other parameters that are considered to identify salt-tolerant genotypes. However, a single trait cannot be relied for evaluating salt tolerance because germination depends upon various other factors studied by Ashraf *et al*. (2006).

Salinity also showed harmful effect on the survival percentage of all aromatic and non-aromatic rice genotypes in present experiment. At all salinity treatments, genotypes DR-92, DR-51 and IR-6 performed better and showed the lowest PROC, whereas the genotypes Khushboo, DR-83, DR-50, DR-52 and Mahek performed meager and showed the highest PROC. Intense reduction in seedling survival and growth is associated with saline environment (Puvanitha and Mahenderan, 2017; Zeng and Shannon, 2000). Roots can be an important trait for the identification of salt tolerant genotypes as these are directly affected by salt concentration (Khan *et al*., 2007).

In this study the root length decreased in all genotypes when exposed to higher salinity levels. The maximum PROC regarding root length was observed in genotypes

Table 4. Effect of salinity on root length and root dry weight of aromatic and non-aromatic rice genotypes grown in solution culture ($NaCl + CaCl_2$ @ 20:1 salt concentrations (mM)

Genotypes	Root length (cm)					Root dry weight (g)				
	T1	T2	T3	T4	Genotype mean	T1	T2	T3	T4	Genotype mean
Sarshar	3.7	3.3	2.8	1.1	2.7	0.043	0.037	0.033	0.023	0.034
Shadab	3.7	3.4	2.9	1.3	2.8	0.035	0.032	0.028	0.023	0.030
Shandar	3.3	3.0	2.5	1.5	2.6	0.025	0.023	0.020	0.012	0.020
Shua-92	3.5	3.1	3.0	2.2	2.9	0.054	0.052	0.049	0.035	0.048
NIA-19A	3.3	3.1	2.7	2.3	2.8	0.048	0.043	0.039	0.035	0.041
NIA-625	3.6	3.3	2.6	1.5	2.7	0.043	0.038	0.033	0.028	0.036
DR-50	2.8	2.5	2.1	1.2	2.2	0.039	0.033	0.028	0.017	0.029
DR-57	3.7	3.1	2.8	0.0	2.4	0.034	0.034	0.030	0.000	0.025
DR-83	3.3	3.1	0.0	0.0	1.6	0.040	0.039	0.000	0.000	0.020
DR-52	3.7	3.2	2.3	0.0	2.3	0.046	0.043	0.037	0.000	0.032
DR-51	3.9	3.5	2.7	2.3	3.1	0.046	0.044	0.039	0.031	0.040
DR-82	3.8	3.7	2.3	1.8	2.9	0.037	0.032	0.029	0.022	0.030
DR-92	4.1	3.8	3.5	2.8	3.5	0.051	0.050	0.042	0.035	0.045
IR-6	3.8	3.7	2.3	2.0	2.9	0.045	0.043	0.037	0.028	0.038
IR-8	5.0	3.8	2.5	1.2	3.1	0.047	0.040	0.039	0.025	0.038
Sada Hayat	3.9	3.5	2.6	1.6	2.9	0.034	0.030	0.027	0.021	0.028
Kanwal-95	5.0	4.0	2.6	1.4	3.2	0.040	0.038	0.036	0.021	0.034
Shahkar	4.0	3.6	2.5	1.8	3.0	0.047	0.044	0.039	0.032	0.041
DR-59	4.0	3.9	2.8	1.5	3.1	0.034	0.031	0.027	0.022	0.029
Mahek	2.6	2.4	2.1	0.0	1.8	0.037	0.030	0.025	0.000	0.023
Khushboo	2.8	2.6	0.0	0.0	1.3	0.033	0.031	0.000	0.000	0.016
DR-62	3.2	2.5	2.0	1.0	2.2	0.041	0.040	0.037	0.017	0.034
DR-66	3.9	3.3	2.8	1.9	3.0	0.045	0.043	0.039	0.024	0.038
DR-67	3.1	2.8	2.4	1.7	2.5	0.047	0.046	0.041	0.034	0.042
DR-63	2.7	2.2	2.2	1.2	2.1	0.046	0.040	0.036	0.024	0.037
Super Basmati	3.2	3.1	2.2	1.9	2.6	0.048	0.042	0.035	0.027	0.038
DR-61	3.7	3.0	2.5	1.4	2.6	0.035	0.030	0.025	0.018	0.027
Latifee	5.1	4.4	3.5	2.9	4.0	0.040	0.038	0.036	0.024	0.035
Treatment Mean	3.66	3.24	2.39	1.41		0.041	0.038	0.032	0.021	
		Genotype (G)		**Salinity**	**G X S**		**Genotype (G)**		**Salinity**	**G X S**
S.E.D		0.11		0.04	0.22		1.47		5.58	2.95
L.S.D (0.05%)		0.22***		0.08***	0.44***		2.91***		11.17***	5.82***

Khushboo, DR-83 and DR-52, whereas minimum PROC was recorded in genotypes DR-92, NIA-19A and Super Basmati. Puvanitha and Mahenderan (2017) has also reported the decrease in root length under saline environment in rice. The reduction in root length with increase in salt-stress might be due to the inhibitory effect of sodium chloride salt (Rahman *et al*., 2001). Similarly, the significant effect of salinity regarding shoot height was observed in all genotypes. A reduction of seedling is a general phenomenon of various crops in saline environment (Hakim *et al*., 2010).

Puvanitha and Mahenderan (2017) also proved the hazardous influence of salinity on shoot height in rice crop especially in salt susceptible varieties. Results from the present experiments showed reduction in shoot and root dry weights of rice significantly with increased salinity treatments. The highest reduction in shoot and root was observed at 120 mM salinity, whereas the lowest reduction was recorded at 40 mM salinity as compared to control treatment. Hakim *et al*. (2014) reported that the reduction in shoot and root dry weight due to decreased per unit photosynthesis leaf area. This

Table 5. Effect of salinity on root shoot ratio and total dry matter production of aromatic and non-aromatic rice genotypes grown in solution culture. The rice genotypes against salinity tolerance on the basis of total dry matter production (g/10 plants)

Genotypes	Root shoot ratio					Total dry matter production				Tolerance at salinity level		
	T1	T2	T3	T4	Genotype mean	T1	T2	T3	T4	T2	T2	T3
Sarshar	2.10	2.03	1.69	1.00	1.70	0.121	0.115	0.105	0.054	T	T	MS
Shadab	2.77	2.40	2.16	0.91	2.06	0.133	0.117	0.100	0.049	T	MT	S
Shandar	3.53	1.35	1.29	1.56	1.93	0.132	0.113	0.088	0.055	T	MT	MS
Shua-92	2.41	2.38	2.26	1.72	2.19	0.185	0.176	0.161	0.12	T	T	MT
NIA-19A	1.96	1.85	1.73	1.49	1.76	0.14	0.125	0.112	0.084	T	T	MT
NIA-625	1.94	1.87	1.80	0.84	1.61	0.128	0.114	0.093	0.082	T	MT	MT
DR-50	2.16	2.02	1.65	1.55	1.84	0.115	0.106	0.086	0.046	T	MT	MS
DR-57	2.34	2.25	2.14	0.00	1.68	0.116	0.111	0.093	0.000	T	T	S
DR-83	1.91	1.84	0.00	0.00	0.94	0.114	0.110	0.000	0.000	T	S	S
DR-52	1.80	1.70	1.62	0.00	1.28	0.129	0.117	0.096	0.000	T	MT	S
DR-51	2.70	2.49	2.33	2.26	2.44	0.172	0.154	0.140	0.120	T	T	MT
DR-82	2.22	2.02	1.85	1.62	1.93	0.118	0.105	0.090	0.060	T	MT	MS
DR-92	2.75	2.65	2.38	2.27	2.51	0.175	0.171	0.144	0.115	T	T	MT
IR-6	2.58	2.43	2.24	2.01	2.31	0.162	0.150	0.118	0.109	T	MT	MT
IR-8	1.65	1.54	1.15	0.73	1.27	0.125	0.114	0.091	0.056	T	MT	MS
Sada Hayat	2.40	2.22	2.08	1.59	2.07	0.116	0.111	0.087	0.067	T	MT	MS
Kanwal-95	2.21	1.95	1.89	1.68	1.93	0.118	0.105	0.097	0.060	T	T	MS
Shahkar	2.50	2.44	1.95	1.88	2.19	0.164	0.154	0.127	0.095	T	MT	MS
DR-59	2.22	2.02	1.85	1.58	1.92	0.11	0.101	0.087	0.055	T	T	MS
Mahek	3.37	2.92	1.86	0.00	2.04	0.143	0.129	0.073	0.000	T	MS	S
Khushboo	3.08	2.96	0.00	0.00	1.51	0.134	0.123	0.000	0.000	T	S	S
DR-62	2.10	1.93	1.68	1.50	1.80	0.125	0.112	0.082	0.047	T	MT	S
DR-66	2.41	1.76	1.66	1.23	1.76	0.153	0.137	0.116	0.088	T	MT	MS
DR-67	2.22	2.10	1.90	1.70	1.98	0.128	0.125	0.108	0.086	T	T	MT
DR-63	1.71	1.64	1.59	1.06	1.50	0.126	0.120	0.102	0.049	T	T	S
Super Basmati	1.74	1.65	1.50	1.30	1.55	0.146	0.130	0.113	0.073	T	MT	MS
~~DR-61~~	~~1.74~~	~~1.68~~	~~1.53~~	~~1.15~~	~~1.52~~	~~0.117~~	~~0.113~~	~~0.091~~	~~0.045~~	~~T~~	~~MT~~	~~S~~
Latifee	2.70	2.66	2.45	1.86	2.42	0.146	0.141	0.121	0.102	T	T	MT
Treatment mean	2.33	2.10	1.72	1.23								
		Genotype (G)			Salinity	G X S						
S.E.D		0.07 (Genotype)			0.02 (Salinity)	0.15 (GXS)						
L.S.D (0.05%)		0.15***			0.05*	0.30***						

Note: T = Tolerant; MT = Moderately tolerant; MS = Moderately sensitive; S = Sensitive

result in inadequate supply of starch needed for shoot growth and decreased turgor, resulting in lower water potential and imbalance supply of nutrients in saline environment.

The shoot/root ratio may also be a good criterion for screening of salt-tolerant crop species. In the current study, a significant difference was observed amongst all genotypes under saline environment as compared to control and this ratio was decreased with increased treatment of salinity. Our findings are in consensus with the findings of Pradheeban *et al.* (2017) that salinity significantly reduces shoot/root ratio due to toxic effects of NaCl salt.

Conclusion

The conclusion is that, the higher concentration of salts inhabited the germination of seeds and effects also on growth of seedlings in various rice genotypes. The dry shoots and roots, ratio yields of all aromatic and non-aromatic genotypes were significantly decreased with increased salinity levels. Compared to other genotypes the DR-92, DR-51, IR-6, Latifee and DR-67 genotypes were less affected by salinity at all treatments. While, the Mahek, Khusboo and DR-83 genotypes are ranked as salt-sensitive in nature, further studies in laboratory and field conditions at other growth and development stages are proposed to establish and classify the salt-tolerant and salt-sensitive genotypes of rice.

Acknowledgment

We are thankfull to the Centre for Bio-Saline Agriculture, Tandojam, Department of Soil Science, Sindh Agriculture University, Tandojam, and Department of Botany, Shah Abdul Latif University, Khairpur for their technical support. We are also grateful to Rice Research Institute Dokri, district Larkana, Sindh, Pakistan and Nuclear Institute of Agriculture Tandojam, Sindh, Pakistan for providing seeds of rice. We are also grateful to the two anonymous reviewers for their valuable comments for the improvement of manuscript.

Conflict of Interest. The authors declare no conflict of interest.

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Pak. j. sci. ind. res. Ser. B: biol. sci. 2021 **64B**(1) 75-80

Seasonal Variation in Microbial Contamination of Various Food Items in Karachi

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(received February 27, 2019; revised September 16, 2019; accepted October 22, 2019)

Abstract. The present study was carried out to investigate the effects of seasonal variation on the microbiological quality of various food items collected from Karachi, Pakistan. A total of two thousand food samples were collected during summer, winter, spring, and autumn season and analyzed for total bacterial count (TBC), total *Coliform* count (TCC), *Fecal coliform* count (FCC), mould and yeast count (MYC) and *Salmonella* spp. The highest percentage of unfit samples was recorded during summer *i.e*. 25.95%, while the lowest value 11.24% and samples were found unfit in terms of total bacterial count during winter. Furthermore, 24.25% of samples were unsatisfactory during the autumn season followed by spring *i.e*. 14.54%. Moreover, findings further demonstrated that MYC was observed higher in all seasons as compared to TBC. None of the samples was found positive for *Salmonella* spp.

Keywords: seasonal variation, microbial contamination, food commodities

Introduction

The basic right of every human being is healthy and nutritions food studied by (Ayala and Meier, 2017). Seasonal variations play a key role in the survival and multi-plication of microbes in food products (Nalepa *et al*., 2018; Suriyasathaporn and Nakprasert, 2012). Seasonal variability not only affects food quality but also the accessibility of food (Zimba *et al*., 2019). Consequently, there are major economic, public, social and environmental consequences. Several findings have shown that seasonal variation e.g. temperature and humidity affect food safety and also have negative effects impacts on local and international food trade (Ali *et al*., 2017).

In Pakistan seasonal pattern is characterised by four seasons, winter (December to February), spring (March to May), summer including rainy season or monsoon period (June to September) and autumn (October to November). The higher temperatures and humidity in summer support bacterial and fungal growth in food commodities (Koluman *et al*., 2017). In fact, for their growth and multiplication, different groups of micro-organism need different ranges of optimum temperature. It has been reported that bacteria, especially in food, prefer to grow in the temperature range between 32 to 43 °C (Mercier *et al*., 2017). In a recent study, it was found that the prevalence of some of the food diseases is higher in summer than in winter seasons (Gong *et al*., 2018; Burkart *et al*., 2011).

In fact, food losses caused negative impacts not only on food quality and safety but on economic development and the environment (Sheahan and Barrett, 2017). Unfortunately, in Pakistan, limited research has been done on food contamination and related topics. Therefore, it is an exigent need to identify those factors that can minimize food losses and have valid information on food quality and safety.

Materials and Methods

Two thousand food samples (raw, cooked, uncooked, and frozen) were collected from different industries of Karachi, Pakistan. They included herbs and spices (492), cereals and cereal products (463), Sugar and confectionaries (190), sauces, pulp and pastes (175), dry fruits (140), fruits and vegetables (fresh/frozen/processed) (119), ready to eat products (106), juices and syrups (68), fat, oil and dressings (52), meat and poultry (52), dry mixes (47), milk and dairy products (42), fish and

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other seafood (33), bakery products (13), egg and egg products (08) (Fig. 1). The samples were taken aseptically in labelled polyethylene bags and carried out for quantitative and qualitative microbiological examination to the laboratory. These samples were collected during a period of about 12 months (2015 to 2016). Frozen samples were stored at -20 °C for less than 24 h and perishable food samples were stored at 0 to 4 °C, while none perishable food samples were stored for less than 24 h at room temperature.

Sample preparation. About 50 g of each sample was placed in the blender jar and added 450 mL of sterile Butterfield's phosphate buffer (pH 7.0) and mixed well, for at least 2 min (this will be 1: 10 dilution). Powder samples were added in small portion, mixed thoroughly after each addition to homogenize the sample. Then serial dilutions were made by transferring of 10 mL of successive dilution to a universal bottle containing 90 mL of sterile Butterfield's phosphate buffer (pH 7.0). In according with specific standard methods described by BAM (Bacteriological Analytical Manual, online, 2006; USFDA, Chapter # 05) these suspensions were seeded in various culture media under incubation parameters. Standard methods were adapted for the detection of aerobic plate count (Andrews *et al*., 2006), mould and yeast count, *Coliform*, *Fecal coliform* (AFNOR/NF BIO 12/20-12/06) and *Salmonella* (ISO 6579:2002; AFNOR BIO 12/01-04/94 protocol).

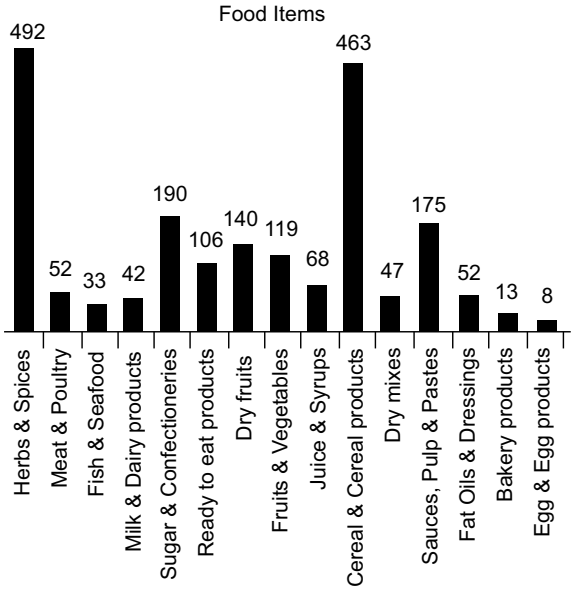

Fig. 1. Frequency of food products analyzed in the present study.

Total bacterial count (TBC). TBC was determined by pour plate method, briefly 1 mL portion from three dilutions *i.e*. 1:10, 1:100 and 1:1000 were transferred to the respective sterile petri plates. Plate Count Agar (Oxoid) was then poured in each petri dish and incubated at 35 °C for 48 h. After incubation colonies were counted in plates having colonies in the ranges of 25-250. The observed values in cfu/mL. The blank control was un-inoculatd plate count agar plate (Smith and Townsend, 1999).

Total *Coliform* count (TCC) and *Fecal coliform* count (FCC). MPN method was employed for the estimation of TCC and FCC. An aliquot of 1 mL of each dilution (1:10, 1:100 and 1:1000) was placed into three sets of 10 mL Lauryl Sulphate Broth (Merck) containing inverted Durham's tubes. These were incubated over 24 and 48 h at 35±0.5 °C. After incubation period tubes were observed for turbidity and gas production. For the confirmation of *Coliform*, tubes with gas and turbidity were sub-cultured into Brilliant Green Lactose bile broth (Merck) and incubated at 35±0.5 °C for gas production at 48±2 h. For the confirmation of *Fecal Coliform*, 10 mL of EC broth (Oxoid) with inverted Durham's tubes was inoculated by a loopful of each suspension. Inoculated tubes were incubated at 44.5±0.2 °C for 24±2 h and examined for gas output (Reddy *et al*., 2009). Total *Coliform* and *Fecal coliform* were calculated from MPN Tables. The blank control was Lauryl Tryptone broth (LTB) (Oxoid). The test tubes with *Escherichia* (E) *coli* were used as a positive control, while *Staphylococcus (S) aureus* were used as negative control.

Enumeration of mould and yeast. 1 mL from the dilutions *i.e*. 1:10, 1:100 and 1:1000 was placed in each petri dish followed by pouring and mixing Dichloran Rose Bengal Chloramphenicol Agar (Merck). After solidification, plates were incubated at 25 °C for 5-7 days. The *Candida* or *Aspergillus niger* were used as a positive control, while *Staphylococcus aureus* were used as negative control (Spangenberg and Ingham, 2000).

Detection of *Salmonella*. The 25 g of homogenized sample was transferred aseptically in a 225 mL of sterile Lactose broth (Merck) and incubated for 24±2 h at 35 °C. After incubation, 0.1 mL of the pre-enriched culture then transferred to 10 mL of Rappaport-Vassiliadis (Merck) medium and again incubated for 24±2 h at 42±0.2 °C. In analogous, 1 mL of pre-enriched culture was inoculated into 10 mL of Tetrathionate Broth (Merck) and incubated for 24±2 h at 43±0.2 °C. Further isolation was carried out on Bismuth Sulphite Agar

(Merck), Xylose Lysine Desoxycholate Agar (Merck) and Hektoen Enteric Agar (Merck). The plates were incubated at 35 °C for 24±2 h and were observed in normal *Salmonella* colonies. For identification, biochemical tests were also carried out. Mini Vidas analyser (bio Merieux) was used for the detection of *Salmonella* antigens. Confirmatory testing was performed by (Kebede *et al*., 2016). For *Salmonella* detection, *Salmonella typhi* were used as a positive control, while *Staphylococcus aureus* or *E. coli* were used as negative control.

Statistical analysis. Mean values were calculated from three replicates. The observed data subjected to analysis of variance and *t*-test by using software (IBM SPSS STATISTICS 20). Significance was considered at P = <0.05.

Results and Discussion

To determine microbial contamination a total of 2000 food samples were collected during different seasons from Karachi region, in summer season 678 samples collected and examined. 176 samples were found highly contaminated and 26 samples for TBC, 78 samples for TCC, 25 samples for FCC and 47 samples for MYC. In autumn season, the total 503 food samples were collected and analysed from which 122 samples were unfit for human consumption as per PS shown in (Table 1), the 16 for TBC, 57 for TCC, 16 for FCC and 33 for MYC. While *Salmonella* spp. were not detected in any sample. During spring season, 330 food samples were collected and analysed. Out of these, 48 samples were declared as unacceptable and 9 samples for TBC, 19 samples for TCC, 9 samples for FCC and 11 samples for MYC. *Salmonella* spp. were also found absent. During winter season 489 food samples were collected and examined, total 55 samples were recorded as unsatisfactory, while 9 for TBC, 20 for TCC, 8 for FCC and 18 for MYC, whereas none of the sample was found to carry *Salmonella* spp. shown in Table 2.

Data clearly showed that the summer season (25.95%), the highest percentage of unsatisfactory samples was found, where herbs and spices commodities were fall into highest percentage of contamination then dry mixes and cereal and cereal products, followed by autumn (24.25%), spring (14.54%) and winter (11.24%). The growth and survival of micro-organisms and temperature fluctuations have already been reported to be directly proportional to each other (Tshikantwa *et al*., 2018). Comparatively higher counts were observed in summer (25.95%) and autumn (24.25%), while lower counts were observed in spring *i.e*. 14.54% and in winter 11.24% (Fig. 2). Statistically, a significant difference (P<0.05) was observed between level of contamination in food samples collected during summer and winter seasons. Various researchers from other parts of the world have also claimed the effect of seasonal variation on the microbiological quality of food items (Nalepa *et al*., 2018; Denis *et al*., 2016; Akil *et al*., 2014; Eltigani *et al*., 2013; Suriyasathaporn and Nakprasert, 2012).

Previously reported studies (Denis *et al*., 2016; Hammond *et al*., 2015) found a higher incidence rate of microbial contamination in food products during the summer compared to the winter season. The same pattern has also been noticed in the present study which suggested that high microbial load in summer could may be due to availability of favourable environmental conditions such as temperature and humidity. In another study, it has been documented that higher ambient air temperature is directly associated with increased incidence of food spoilage (Dominianni *et al*., 2018; Moh *et al*., 2017). For instance, in Karachi summer season (June to September) has been characterized by temperature in between 30-35 °C along with heavy rainfall. Therefore, combination of warm weather and sufficient humidity support the growth of variety of microbes.

Findings of the current study revealed the lowest percentage of unsatisfactory food samples during winter season. Like other regions winter season has been characterized by low temperature, humidity and precipitation in Karachi. It has been reported that low temperatures during winter season is a limiting factor for variety of microbes (Kim and Ndegwa, 2018). The findings by (Duvenage and Korsten, 2016; Ashenafi, 2012) also justified that low temperature during winter season negatively affects microbial growth.

It has been noticed that the number of samples contaminated with MYC was higher in all seasons as compared to the number of samples contaminated with bacteria. It is evident in Fig. 3 that in summer 6.93% samples were found positive for MYC, while 3.83% for bacteria. Likewise, 6.56% samples were found positive for MYC, whereas 3.18% for bacteria in autumn. In winter 3.68% and 1.84% samples were found positive for MYC and bacteria respectively and in spring moulds and yeasts and bacteria were detected in 3.33% and 2.72% samples respectively. Similar findings were also reported by Snyder and Worobo (2018), suggested that since fungal spores rapidly dispersed *via* air and water and survive over a wide range of environmental conditions. Moreover, some fungal species persist under

Table 1. Standard limits for food commodities as per Pakistan standards (PS)

Food items description	Pakistan standard limits (cfu/g or mL)									Pakistan standard Reference no.
	TBC			TCC			MYC			
	S	b	U	s	B	U	s	b	U	
Herbs and spices	10^4	$>10^4$ - $<10^6$	$>10^6$	10^2	$>10^2$ - $<10^3$	10^3	10^2	$>10^2$ - $<10^4$	10^4	3741-1996
Milk and dairy products	103	$>10^3$ - $<10^4$	$>10^4$	10^2	$>10^2$ - $<10^3$	10^3	10	>10 - $<10^2$	10^2	2835-1990, 363-1991, 2832-1-1990, 2027-1988
Chocolate confectionaries	10^3	$>10^3$ - $<10^6$	10^6	1.8	>1.8 - $<10^2$	10^2	10^2	$>10^2$ - $<10^3$	10^3	4715-2001, 4716-2001, 4557-2000/5243
Sugar confectionaries	10^4	$>10^4$ - $<10^6$	10^6	1.8	>1.8 - $<10^2$	10^2	10	>10 - $<10^2$	10^2	4717-2001
Egg & egg products	2.5×10^4	$>10^4$ - $<10^5$	10^5	10	>10 - $<10^3$	10^3	10	>10	-	FDA Circular 2013-010
Flour	10^2	$>10^2$ - $<10^5$	10^5	10	>10 - $<10^2$	10^2	10^2	$>10^2$ - $<10^4$	10^4	1931-2013
Baked goods	10^4	$>10^4$ - $<10^6$	10^6	50	>50 - $<10^3$	10^3	10^2	$>10^2$ - $<10^4$	10^4	761-2001, 382- 1964/ PS 4840
Chicken meat	5×10^3	$>10^3$ - $<10^7$	10^7	10	>10 - $<10^2$	10^2	---	---	---	4726 2001
Meat	10^4	$>10^4$ - $<10^5$	10^5	10	>10 - $<10^2$	10^2	---	---	---	2861-1990, 2826- 1990, 2827-1990, 2988 -1991,
Fats, oil and dressings	10	>10 - $<10^2$	10^2	---	---	---	10	>10 - $<10^2$	10^2	358-1997/2858,
Cereal and cereal grains	10^2	$>10^3$ - $<10^6$	10^6	10^2	$>10^2$ - $<10^4$	10^4	10^2	$>10^2$ - $<10^4$	10^4	1931-2013, 3342-1993, 154-1962
Dry mixes	10^4	$>10^4$ - $<10^6$	10^6	10	>10 - $<10^3$	10^3	10^2	$>10^2$ - $<10^4$	10^4	FDA Circular 2013-010
Dried fruits	$<10^5$	$>10^5$ - $<10^6$	10^6	3	>3 - <11	11	10^2	$>10^3$ - $<10^4$	10^4	1689-1985/2013
Cooked food/ready to eat	$<10^4$	$>10^4$ - $<10^5$	$>10^5$	10	>10 - $<10^2$	10^2	10	>10 - $<10^3$	10^3	FDA Circular 2013-010
Fruits and vegetables	---	---	---	3	>3	>10	10^2	$>10^2$ - $<10^4$	10^4	3945-1997, 510-1996, 520-1999, 4563-2000, 1923- 1987, 4569-2000
Juices	102	$>10^2$ - $<10^5$	105	01	>1	>10	10	>10 - <50	50	527-1992
Syrups	104	>104	5×104	10	>10	10^2	-	-	-	3114-1991
Sauces, pulp and pastes	10	>10 - $<10^2$	10^2	10	>10 - $<10^3$	10^3	10	>10 - $<10^2$	10^2	3947-1997, 512-1964
Fish and other seafood	5×10^5	$<10^5$ - $<10^7$	10^7	11	>11	500	---	---	---	2834

Table 2. Data showing microbiological analysis of food samples with respect to seasonal variation

Seasons	n	Microbiological parameters									
		TBC		TCC		FCC		MYC		*Salmonella*	
		U	f	u	F	u	f	u	f	u	f
Summer, Jun.-Sep.	678	26	652	78	600	25	653	47	631	00	678
Autumn, Oct.-Nov.	503	16	487	57	446	16	487	33	470	00	503
Winter, Dec.-Feb.	489	09	480	20	469	08	481	18	471	00	489
Spring, Mar.-May	330	09	321	19	311	09	321	11	319	00	330

Key: n = total no. of samples analysed; u = unfit samples; f = fit samples; TBC = total bacterial count; TCC = total *Coliform* count; FCC = *Fecal coliform* count; MYC = mold and yeast count.

the most extreme physico-chemical processing employed in commercial food production. Anwer *et al*. (2017) also supported the significant role of fungi in the spoilage of various processed foods. It is probably due to the proliferation of fungi in foods containing limited moisture content.

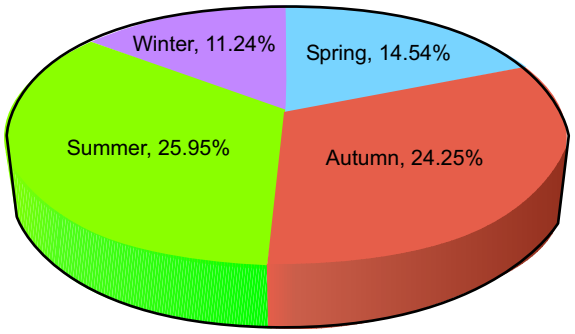

Fig. 2. Frequency of unsatisfactory samples in terms of seasonal variation.

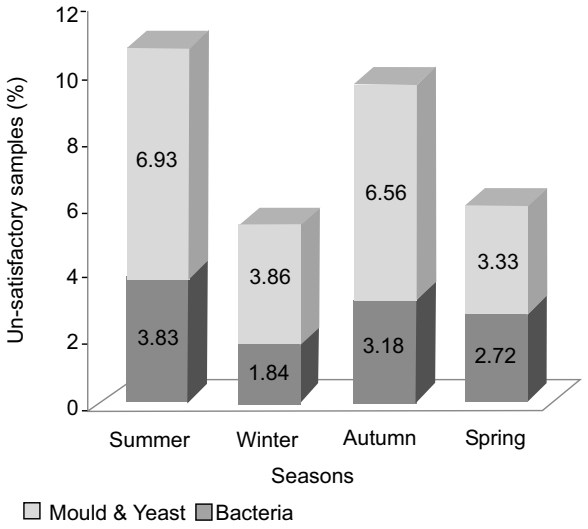

Fig. 3. Bars showing occurance of bacterial and fungal contamination in food samples during four seasons.

Keeping in view, the above findings it has been verified that the seasonal variation significantly affect microbial growth and multiplication. In odour to keep an eye on rising economical and health risks in terms of food preparation and consumption, this baseline study will provide sufficient data to step ahead the issues of food quality and safety.

Conflict of Interest. The authors declare no conflict of interest.

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Pak. j. sci. ind. res. Ser. B: biol. sci. 2021 **64B**(1) 81-86

Evaluation of *Phyllanthus niruri* L. from Malaysia for *In-vitro* Anti-Urolithiatic Properties by Different Solvent Extraction

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(received January 29, 2019; revised February 21st, 2019; accepted February 26, 2019)

Abstract. The *Phyllanthus niruri* is traditionally used for curing of kidney disorders and urinary stones in Malaysia. Hence the current work was aimed to evaluate the effect of different solvents extract (n-hexane, ethyl acetate, methanol and water) of *P. niruri* for *in vitro* anti-urolithiatic properties in terms of inhibition activity on CaOx by using the rate of CaOx aggregation assay and dissolution of calcium oxalate (CaOx) crystal by using titrimetry method. Cystone was used as positive control. The effects of cystone on slope of nucleation and aggregation as well as growth of CaOx were evaluated spectrophotometrically. The highest yield percentage of *P.niruri* was occupied by methanol (5.74 %). The maximum inhibition against aggregation of CaOx crystals was also occupied by methanol (66.67 % ± 1.61) and was comprised with alkaloid, steroid, terpenoid and tannin. Dissolution effect on calcium oxalate crystals indicates that the aqueous extracts of *P. niruri* was found to be more effective in dissolution of CaOx with 63.33 % ± 1.44. *P. niruri* significantly ($P < 0.05$) inhibited the slope of nucleation and aggregation of CaOx crystallization, and reduced the crystal density. The results of the present study confirmed that *P. niruri* leaves can be used as remedial mediator for urolithiasis. However, further studies are required for isolation and identification of active constituents and their *in-vivo* confirmation.

Keyword: crystallization, dissolution, nucleation, *P. niruri,* anti-urolithiatic.

Introduction

Urolithiasis (from Greek oûron, " urine" and " stone") is a condition in which urinary calculus is formed or located anywhere in the urinary system or stones are formed in the kidney, bladder or ureters (Sharma *et al*., 2016). Different phytochemical events begins when the formation of kidney stone occurs like crystal nucleation, aggregation and end with retention within the urinary tract. Among the several types of kidney stones, the most common are calcium oxalate representing up to 80% of the analyzed stones. Calcium containing stones may be in the form of pure calcium oxalate (50%) or calcium phosphate (5%) and a mixture of both (45%) followed by magnesium phosphate (15-20%), uric acid (10%) and cystine (1%) (Singanallur *et al*., 2017). There are numerous methods had been reported to reduced or break the kidney stone. Traditional method of treatment is being reported from plants which are the most effective. Plants based on traditional knowledge can lead to the discovery of new drug and development of pharmacologically important products for human health care (Pauzi *et al*., 2018; Subramoniam, 2014). Almost, 80% of the world's population depends on the conventional medicine to cure most of their diseases (Gul *et al*., 2019; Kennedy, 2005). There is a number of plants which show promising anti-urolithiatic activity (Ram *et al*., 2015). Nowadays, these conventional remedies have become more popular because they are very efficient, have low side effects and reduce the reformation of stone. Usually, the decoction and infusion

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methods are used for extraction that is good way for extracting compounds of various plants.

Although water decoction method is still using, even this method abundantly used huge volume of water. Moreover, there are some dis-advantages associated with water such as water that gives an excellent growth for microbes and this condition leads to microbial contamination to the samples. Moreover, it will promote hydrolysis and enzymatic degradation in the plant sample (Azmir *et al*., 2013). In addition, water also attract to an extract along with polar compounds which could obstruct in the identification and quantification (Bandar *et al*., 2013). Furthermore, the large volume of hot water usually means that the plant sample will exposure to unpleasant taste for longer period (Bone and Mills, 2013).

The *Phyllanthus niruri* is the member of the family Euphorbiaceae, and because of its speciality commonly known as as "stone-breaker" (Kieley *et al*., 2008). The habitat of the plant is moist, shady places, rock and some time epiphytes. The morphology of the plants states that leaf blade rounded to complement the elongated egg and green fruit. It can grow up to 60 cm. furthermore, the tastes of *P. niruri* is bitter, cool, and as an astringent (Dalimartha, 2008).

Wang *et al*. (1995) identified the bioactive compounds like alkaloids, coumarins flavonoids, lignans, polyphenols, saponins tannins and terpenoids from different parts of *P. niruri*. Furthermore, Bagalkotkar *et al*. (2006) stated that 50 different bio-active compounds were identified from the *P. niruri*, including flavonoids, alkaloids, triterpenes and lignans. This is the proved that this plant diverse photochemical contents in different experimental studies. Alkaloid and triterpenes reported by many research as inhibited the cytotoxicity activated by calcium oxalate (Malini *et al*., 2000). Therefore, the current study will focus on the analytical methodologies, which include the extraction and its application as anti-urolithiatic activity.

Materials and Methods

Sample collection. The grinded leaves of *P. niruri* were purchased from Seri Subah Agrofarm, Negeri Semblian, Malaysia.

Sample preparation. The grinded plant samples were kept in the room temperature and dry place to maintain them in dry condition. The moisture content of the samples were measured and maintained at consistently about not more than 10 % (Azwanida, 2015). Cystone was used as positive control while, distilled water was used as negative control.

Extraction process. The extraction method was followed by Fermeglia (2008) with slight modification. The plant samples were extracted by unlike non-polar solvents to polar solvents that are *n*-hexane, ethyl acetate, methanol and water. The extraction method was maceration using. The experiment was carried out in three replicates. The following equation used to calculate extraction yield:

$$\text{Total extract yield, Y (\%)} = \frac{\text{Total mass of extraction}}{\text{Total mass of sample}} \text{ x } 100$$

Phytochemicals analysis of the plant samples. Phyto-chemical analysis was performed by standard method followed by Tiwari *et al*. (2011). All extracts used in these assays were 1 mg/mL in concentration.

Evaluation of anti-urolithiatic properties (*in-vitro*). ***Inhibition activity of plant extracts against calcium oxalate (CaOx) crystal by aggregation assay.*** The aggregation assay was done followed by Hess *et al*. (2000) with slight modifications. In addition, the inhibition rates of CaOx aggregation by the extracts were compared with the standard drugs, Cystone. CaOx crystals solution was prepared by using 10 mM calcium chloride dihydrate and 1.0 mM sodium oxalate, containing 200 mM NaCl and 10 mM sodium acetate trihydrate. All tests were conducted at 37 °C and 5.7 pH. For crystallization of CaOx, 25 mL of calcium oxalate solution was shifted to a beaker and placed in a constantly stirring hot plate magnetic stirrer. Next to it added 1 mL of plant extract (1 mg/mL)/ Cystone (1 mg/mL)/distilled water. The formation of the turbidity results immediately after the addition of 25 mL of sodium oxalate solution. The measurement of turbidity formed in terms of absorbance at 620 nm in UV-Vis spectrophotometer. It was started continuously for ten minutes after the mixing of the chemicals. In fact, the turbidity of solution increased indicates the nucleation process, and then decreased after some time which indicates the aggregation process. This experiment was done in three replications. The percentage inhibition rate of CaOx aggregation was calculated according (Sharma *et al*., 2016).

$$\text{Inhibition \%} = [1- (Si/Sc)] \text{ x } 100$$

where;

Sc = slope of aggregation without inhibitor (negative control); Si = slope of aggregation in the presence of inhibitor (positive control/ plant extracts)

Estimation of calcium oxalate by titrimetry method. Calcium oxalate (10 mg) and plant extract or Cystone (100 mg) was weighed respectively, and packed together in the semi-permeable membrane and carefully sutured. Then, it was allowed to suspend in a conical flask containing 100 mL of 0.1M TRIS buffer. The conical flasks were kept at room temperature for seven to eight hours. The remaining contents in the semi-permeable membrane is transferred into a beaker. Next, 1N sulphuric acid (2mL) was added and titrated with $KMnO_4$ until a light pink colour appeared (Dwivedi, 2016). Consequently, 1 mL of 0.9494 N $KMnO_4$ equivalents to 0.1898 mg of calcium.

% dissolved of calcium = [(C-T)/C] x 100

where;

C = precipitate of calcium oxalate remained in control (mg); T = precipitate of calcium oxalate remained when test solution was used (mg).

Statistical analysis. All the experiments were conducted in triplicate and the data were presented as mean values and standard deviation. One way ANOVA applied on data using IBM SPSS Statistics software (Version 20.0, USA) with the level of significant $P< 0.05$.

Results and Discussion

Yields of extraction. As shown in Table 1, the effect of different solvents were studied in terms of the extraction yield. The solvents were selected based on their polarities. Polarity of a solvent plays a considerable role in the extraction process (Ahmad *et al*., 2017).

Based on the result, the highest yield percentage of P.niruri was occupied by methanol (5.74 %) followed by water (2.15%), ethyl acetate (1.46 %), and lastly n-hexane (0.98 %).

Table 1. The percentage yield of herbal plant extracts

Herbal plant	Type of solvent	Mass of sample (g)	Mass of extract (g)	Yields (%)
Phyllanthus niruri	*n*-hexane	50	0.49	0.98
	Ethyl acetate	50	0.73	1.46
	Methanol	50	2.87	5.74
	Aqueous	50	1.08	2.16

Consequently, different solvents exhibited different yield percentage for each plant samples. The results revealed that solvents yield wide range of extraction (0.98 -5.74%). Among all of the solvent used, methanol exhibited the highest percentage of yields at the maximum percentage of 5.74%. This result was similar to Kotze *et al*. (2002) which reported that methanol shown to be the best extraction solvent for Combretum erythrophyllum as compared with other extraction solvents. Similar findings have been observed in other studies done by Suleiman *et al*. (2010) which reported that hexane extract was found to be the lowest amount of extract yielded from Kirkia wilmsii.

Phytochemical associated with anti-urolithiatic properties of plant extracts. The results of phyto-chemical screening in Table 2 revealed that the presence of alkaloid, steroid, terpenoid, tannin, and saponin in plant extracts. However, based on the result obtained, the amount of detectable phytochemicals in every solvent extract is different from each other. This might be due to the different polarity of solvents could selectively extracts different type of phytochemicals (Dailey and Vuong, 2015; KV *et al*., 2014; Chavan *et al*., 2013; Rebey *et al*., 2012). Different type of phytochemicals that are present in each extract might have some positive contribution to anti-urolithiatic effect against calcium oxalate crystals either in term of inhibition or dissolution properties.

Evaluation of anti-urolithiatic properties (*in-vitro*). ***Inhibiting effect of P. niruri on calcium oxalate crystals.*** The inhibition percentage of *P.niruri* extracts was shown in Table 3. The highest inhibition percentage of *P. niruri* extract against aggregation of CaOx crystals was occupied by methanol with percentage of 66.67 % ±

Table 2. The amount of detectable phytochemical of *P. niruri* extract

Type of solvent	Alkaloid	Steroid	Terpenoid	Tannin	Saponin
n-Hexane	++	++	+	-	-
Ethyl acetate	-	++	-	-	++
Methanol	+	+++	+	+	-
Aqueous	-	+	-	+	++

+ = indicates present; '-' = indicates absent; +++ = indicates phytochemicals in high amount; ++ = indicates phytochemicals in good amount; + = indicates phytochemicals in trace but detectable amount.

1.61 and was comprised with alkaloid, steroid, terpenoid and tannin. The studies regarding the phytochemicals in *P.niruri* were proven by Calixto *et al*. (1998) and Narendra *et al*. (2012) which reported that many bio-active compound from this plant have been identified which includes alkaloids, tannin, steroids and triterpenes.

Meanwhile, the second highest percentage of inhibition was hexane extract with 53.68 % ± 2.11 which also contain the same phytochemical with methanol extract but differ in detectable amount. Moreover, aqueous and ethyl acetate extract of *P.niruri* showed quite low inhibition activity compared to methanol (29.12 % ± 1.22) and *n*-hexane (18.95 % ± 1.06). The significant different (P>0.05) between these two values was probably due to the absence of alkaloid and terpenoid in both extracts.

Dissolution of calcium oxalate crystals by titrimetry assay. This study evaluates the anti-urolithiatic activity by dissolving the artificial CaOx packed in semi permeable egg with the help of different solvent extracts of *P. niruri*. The work was performed by using *in-vitro* anti-urolithiatic model for calculating percentage dissolution of CaOx crystals. The amount of CaOx dissolved was nominated as the indicator to evaluate anti-urolithiatic activity. The results for the dissolution percentage of CaOx by plant extracts and standard are shown in Table 4. The amount of CaOx dissolved with standard drug was 73.33 % ± 3.82 which is the highest percentage as compared to plant extracts. Consequently, all extracts showed their ability to dissolve the amount of CaOx inthe range from 65.83 % to 36.67 %.

Based on the phytochemical screening, the ability of dissolving activity of plant extract on CaOx crystals could be carried out effectively with only minimum amount as compared to inhibiting activity (Fig. 1). This is in agreement with similar finding reported by Dwivedi *et al*. (2016), conclusively revealed that Colocasia leaves show good anti-urolithiatic activity by dissolving the CaOx crystals even at low amount of phytochemicals.

Table 3. The percentage of inhibition on rate of CaOx aggregation by plant extract and standard drug, cystone.

Herbal plant/ Standard drug	Type of solvent	Inhibition percentage (%) (Mean ±Standard Deviation)
Cystone	-	92.28 ± 0.61a
Phyllanthus niruri	*n*-hexane	53.68 ± 2.11d
	Ethyl acetate	18.95 ± 1.06h,i
	Methanol	66.67 ± 1.61c
	Aqueous	29.12 ± 1.22g

[a, b, c,,,] Values designated with different alphabets are significantly different from each other.

Table 4. The percentage of dissolution on CaOx crystals by plant extract and standard drugs, cystone.

Herbal plant/ Standard drug	Type of solvent	Dissolution percentage (%) (Mean ±Standard Deviation)
Cystone	-	73.33 ± 3.82a
Phyllanthus niruri	*n*-hexane	48.33 ± 3.82f,g
	Ethyl acetate	53.33 ± 5.20d,e,f,g
	Methanol	55.00 ± 0.00c,d,e,f
	Aqueous	63.33 ± 1.44b,c

[a, b, c,,,] Values designated with different alphabets are significantly different from each other.

Dissolution effect of *P. niruri* on calcium oxalate crystals. The result shows in Fig. 2. indicates that aqueous extracts of *P. niruri* was found to be more effective in dissolution of CaOx with the percentage of 63.33 % ± 1.44. This result was followed by methanol (55.00 %), ethyl acetate (53.33 % ± 5.20) and lastly *n*-hexane extract (48.33 % ± 3.82).

Similar to previous studies, aqueous extract of *Melia azedarach* was studied in male albino Wistar rats against ethylene glycol-induced nephro-lithiasis and this extract has been shown to reduce urinary calcium, oxalate, phosphate and urinary magnesium levels and urinary

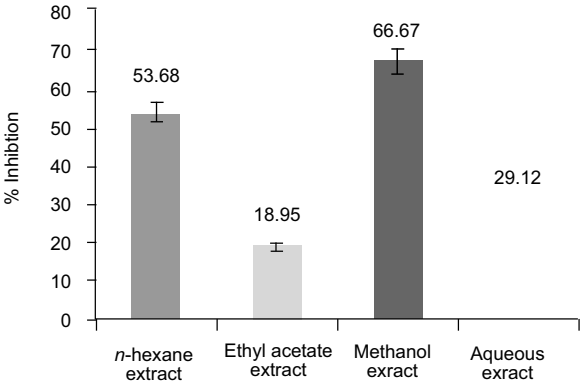

Fig. 1. CaOx inhibition activity of four solvent extracts of *P.niruri*.

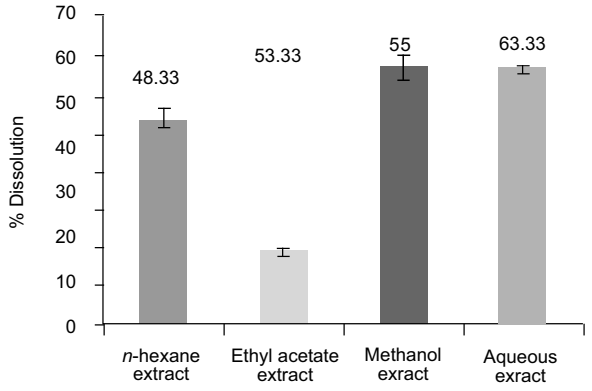

Fig. 2. CaOx dissolution activity of four solvent extracts of *P. niruri*

volume (Garimella *et al*., 2001). Moreover, the aqueous extract of C. Spiralis used at a daily dose of 0.25 and 0.5 g / Kg for 4 weeks reduced the growth of calcium oxalate calculus in the urinary bladder of rats significantly (Viel *et al*., 1999). This indicates that the aqueous type of solvent was capable of extracting various plants effectively and can positively act as anti-urolithiatic agent.

Conclusion

Based on result of extraction yield of all extracts, it has been found that the highest percentage was demonstrated of *P. niruri* extract which obtained by using methanol while the lowest yield percentage was obtained by using n-hexane as the extraction solvent. *P. niruri* extract contains different type of phytochemicals depending on the polarity of the solvent used. According to overall result of phytochemical screening, alkaloids are found to be abundant in hexane extracts while most saponins are contained in water extracts. This result might be affected by the polarity of phyto-chemicals and solvents used. Therefore, the ability of all extracts of *P. niruri* to inhibit and dissolve CaOx crystal might be beneficial in the treatment of urolithiasis in the future. However, there is a need of further scientific investigation and experimental proofs to support these preliminary findings. Besides, an additional work can also be carried out to isolate, purify and characterize bioactive compounds and to identify their possible mechanism of action.

Acknowledgement

The research is funded by the Ministry of Education Malaysia under Fundamental Research Grant Scheme (FRGS) Vot No. 1646 and partially sponsored by Universiti Tun Hussein Onn Malaysia.

Conflict of Interest. The authors declare no conflict of interest.

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Pak. j. sci. ind. res. Ser. B: biol. sci. 2021 **64B**(1) 87-93

Production and Characterisation Partial Lipase of *Bacillus halodurans* Cm1 Mutant for Biodetergen

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(received October 25, 2018; revised March 23, 2019; accepted March 26, 2019)

Abstract. This study aims to produce lipase of the *Bacillus halodurans* CM1 mutant and its assess partial characteristics, performed in Bora and Bora modified medium. The purification was conducted using Ultrafiltration (UF), ammonium sulfate (AS) and polyethylene glycol (PEG). Results revealed that the highest purity lipase of *B. halodurans* CM1 mutant was 1.49-fold from the UF-AS-dyalisis, with a molecular weight of 35.7-37.4 KDa. The optimum condition of lipase enzyme was achieved at pH 7 and temperature 50 °C, relatively stable at pH 7-8 and temperature 30-70 °C. Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+} and K^{+} ions of concentrations, 1 mM to 10 mM increased enzyme lipase activity. The Km value was 0.23 mg/mL and Vmax 4.07 U/mL. Lipase was stable with the addition of a detergent concentration of 1-2% (69.60-57.10%), and with the washing test, the enzyme capable of hydrolyzing oil on cloth is 8.40%.

Keywords: *B. halodurans*, CM1 mutant, characterisation, lipase

Introduction

Lipase is an enzyme capable of catalysing hydrolysis and long chain synthesis of acylglycerols (tri-acylglycerol acyl-hydrolases) (EC 3.1.1.3), Andualema and Gessesse (2012). It is widely used in scientific testing i.e. cosmetic production, medical diagnosis, chemical analysis and bio-detergent industry, Cherif *et al*. (2011).

Bacteria are used to manufacture enzymes they produces a high degree of activity, neutral or alkaline optimum pH, as well as thermostable. Thermophile bacteria are the source of thermostable enzymes, and proteins derived from thermophilic micro-organisms are useful in biotechnology applications due to the high stability. Stability of lipases at high temperatures have a high reactivity, where an increase in substrate and product solubility, as well as a decrease in the viscosity present the risk of contamination. The bacteria of the genus *Bacillus* are capable of producing lipase (Balan *et al*., 2012; Deive *et al*., 2012).

Preliminary research conducted by the BPP Technology team, the bacteria isolated from hot springs, Cimanggu, west Java, had a molecular identification with 16SrDNA as a *Bacillus halodurans* CM1, with a ability to generate extra cellular enzymes, Ulfah *et al*. (2011). The production of lipase by *B. halodurans* CM1 was carried out at pH 9.00 and temperature 50 °C (Unpublished data).

The study with *B. halodurans* CM1 mutated with gamma radiation at doses of 0.1-0.4 KGy was performed according to the Internal Report (2016), however the lipase activity was obtained lower than the mutation resulted using N-methyl-N'- nitro-N-nitrosoguanidine (NTG) with a concentration of 0.1 mg/mL and incubation of 1-3 h. The NTG mutation resulted in *B. halodurans* CM1 gave the highest lipase activity was 5.13 U/mL.

Lipases applied have a wide ranged of pH and temperature stability, good selectivity and specificity to various substrates, and be easy to isolated. Enzyme characterisation needs to be carried out to understand the potential and be applied on an industrial scale Djafar *et al*. (2010).

The aim of this study is to produce lipase of *B. halodurans* CM1 mutant and to test their pH, temperature, metal ion and detergent. The calculation of molecular weight was performed and the kinetic enzyme calculated by using the Lineweaver-Burk equation. The application test for the lipase mutant was performed by assessing the stability of lipase, in addition to the detergent and enzyme ability test in hydrolysing the oil on cloth and the washing test.

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Materials and Methods

Materials commonly used in the bioprocess of lipase production include; lipases that have been concentrated with stirred-cell ultrafiltration (Uicon) [Amicon], UF-ammonium sulfate (USA) [Merck], UF-polyethylene glycol (PEG) [Merck]; SDS-PAGE [Bio-Rad], and metal ions (1 and 10 mM $MnSO_4$ [Merck]; 1 and 10 mM $ZnSO_4$ [Merck 1 and 10 mM]; $CaCl_2$ [Merck]; 1 and 10 mM KCl [Merck]; 1 and 10 mM $MgSO_4$ [Merck] and $FeSO_4$ [Merck].

Lipase production. To build a seed culture, one loop of bacteria was in-oculated on liquid LB medium pH 9.00 with a working volume of 50 mL seed culture in Erlenmeyer 250 mL, agitation 200 rpm, a temperature 50 °C and incubated for 18-24 h.

Ten percent of the seed cultivation was used as the total starter LB medium pH 9.00 with a working volume of 100 mL in 500 mL Erlenmeyer, which were then incubated at 50 °C, agitation 200 rpm, until optical density (OD) reached 0.6-0.8 (2-4 h), before being inoculated into 10% of the total out put medium volume.

Shake culture of production medium Bora and Bora (2012) were modified with 0.5% PO and 0.09% $CaCl_2$ incubated in 3 L Erlenmeyer with a working volume of 500 mL (total working volume 1 L), agitation 200 rpm at 50 °C for 18 h. After 18 h, the cultures were harvested by mixing the two, and centrifuged at 3800 rpm for 30 min at 4 °C. The supernatant was taken as a crude enzyme (Ghaima *et al*., 2014; Mokodongan, 2013).

Assay for lipase activity. The lipase activity test was conducted based on a modification from Li *et al*. (2014). The substrate was made of 25% olive oil, 1.50% polyvinyl alcohol (PVA), and homogenised with reverse osmosis (RO). A substrate of 5 mL was taken and added to 0.05 M tris-HCl buffer solution of pH 8.00 of 4 mL, then 1 mL of enzyme. Incubation was performed at 37 °C, 150 rpm for 20 min, before the sample was added with 5 mL of methanol, titrated with 0.05 M NaOH. The amount of enzyme needed to release one µmol of free fatty acid per minute under the experimental conditions were defined as one unit of lipase activity.

Partial purification. ***Stirred-cell ultrafiltration (UF).*** The 30 KDa filter membrane was installed in the UF device, and 30 psi of pressure was applied. The membranes were pre-treated, followed by 200 mL of crude enzymes being inserted into the tank at 100 rpm. Sampling was performed on crude enzymes prior to the UF process and at 10× concentration, according to modification of Syed *et al*. (2010).

Ammonium sulfate precipitation. The enzyme with a stratified fractionation (20-80%), was slowly added to ammonium sulfate when mixed with the magnetic stirrer. Lipase 10 mL was prepared in a beaker and ammonium sulfate was added to the enzyme based on the ammonium sulphate Table. Ammonium sulphate of 1.07 g was slowly added into the enzymes, where it was mixed until dissolved, then the enzymes were incubated for 1 h. The enzymes were centrifuge at 3800 rpm for 30 min. The precipitated was added with 5 mL of 0.05 M tris-HCl buffer pH 7.00 for dialysis and the supernatant of the centrifugation product was calculated in volume to be used for the subsequent fraction. Membranes for dialysis were first pre-treated, Pratama (2015), one side of the membrane was tied, and the precipitated of ammonium sulfate dissolved with the buffer solution was poured into the dialysis membrane, the other side was also tied. The membrane was then immersed in 0.05 M tris-HCl buffer pH 7.00 with 1 L, rotating at 70 rpm at 18-20 °C overnight (18-24 h). Samples after dialysis, tested for activity and protein levels, Borkar *et al*. (2009).

Polyethylene glycol (PEG). Pre-treatment was performed on 12 KDa cellophane membrane, fastening on one side and filling in 10 mL enzymes sample on the other side for subsequent binding. A container already dusted with PEG 20 KDa was prepared, and the sample containing membranes were put in a container and then sprinkled with PEG until all the membranes had been coated and conditioned at 4 °C. The enzyme was concentrated until the retentate volume reached half the initial volume.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide gel was made using the mold, and after the gel was formed, it was removed and installed in the electrophoresis device tank. The 1× running buffer was poured into the tank until the specified limit. Each 12 µL sample was added with 5×3 µL sample buffer. The samples (15 µL) and markers (5 µL) were boiled in water for 1 min. Samples and markers were further fed into electrophoretic gel wells and the electrophoresis device was given a voltage of 150-200 V. After the process was complete, the gel was released and rinsed with miliQ, before being soaked and heated in a microwave for 1 min, repeated three times. The gel was then immersed in page blue and

heated in a microwave for 30 seconds, before it was incubated on top of thermo shaker for 1 h, at 300 rpm, room temperature (25-30 °C). After 1 h, the gel was rinsed and immersed in RO, and incubated overnight (18-24 h). The molecular weight was estimated by measuring the distance of the sample migration marked by the band and the distance of the sample tracer migration when electrophoresed (the bottom of the gel) as illustrated by Muchtaromah *et al*. (2012).

Profile of pH and lipase temperature. Determination of pH profile lipase of *B. halodurans* CM1 mutant was performed using an enzyme activity test at different pH levels (pH 6, 7, 8, 9, 10, 11 and 12). It was performed by titration method Li *et al*. (2014) with modification, and the buffer was used, ie 0.05 M phosphate buffer pH 6 and 7, 0.05 M Tris-HCl buffer pH 8 and 9, and 0.05 M glycine-NaOH buffer pH 10, 11 and 12. Incubation of the activity test at temperature 50 °C, 150 rpm, was conducted for 20 min. The profile temperature of the enzymes were determined at the optimum pH obtained with the determination of pH profiles consisting of varying temperatures (30, 40, 50, 60, 70 and 80 °C).

Stability of pH and lipase temperature. The effect of pH on stability lipase of *B. halodurans* CM1 mutant was measured by incubating the enzyme in pH buffer 6, 7, 8, 9, 10, 11 and 12 at room temperature for 90 min, then sampling each activity. The effect of temperature on the stability of the enzyme was measured by incubating for 1.5 h (sampling every 30 min) at 30, 40, 50, 60 and 70 °C and then tested its activity, as modified from Li *et al*. (2014).

Influence of metal ion. The influence of metal ions on lipase activity of the *B. halodurans* CM1 mutant was determined by testing enzyme activity containing 1 mM and 10 mM Mn^{2+}, Zn^{2+}, Ca^{2+}, K^{+}, Mg^{2+} and also Fe^{2+} (Mokodongan, 2013; Ghori *et al*. 2011).

Km and Vmax. The rate of lipase in binding to the substrate was determined by analyzing the Km and Vmax values, obtained by testing the enzyme activity at different concentrations of titration substrates. Variations of the substrate concentration used were 1, 2, 8, 12, 16 and 20%. The data of the enzyme activity test results were then plotted by the Lineweaver-Burk equation, Ratnayani *et al*. (2015) modified.

Stability in detergents. The stability of the enzyme to the addition of detergent was measured by the modified method of Cherif *et al*. (2011), where incubation of the lipase of *B. halodurans* CM1 mutant was mixed with added detergent concentrations of 1, 2, 3, 4 and 5% at 37 °C, 180 rpm for 60 min, then its enzyme activity at pH and optimum temperature were teste.

Washing test. WA washing test was performed based on Li *et al*. (2014) method: cloth was cut in 4×4 cm squares and soaked in boiling chloroform for 5 min. The cloth was then dried overnight at room temperature, and then weighed (Wa). The cloth was dropped on both sides by oil dissolved in acetone (100 µL/mL), and weighed again. It was then dried for 15 min at room temperature, immersed in an enzyme dissolved with 0.05 M phosphate buffer pH 7 (1:1), and incubated at 30 °C, 180 rpm for 1 h. The cloth was dried overnight at room temperature then weighed (Wc):

$$\text{Oil lost (\%)} = \frac{\text{Wb - Wc}}{\text{Wb - Wa}} \times 100$$

Results and Discussion

Lipase purification. The basic lipase activity of *B. halodurans* CM1 mutant increased from 9.50 U/mg to 10.38 U/mg following the concentration of stirred-cell ultrafiltration (UF). The results were comparable to the report of Balan *et al*. (2012) study, in that the ultrafiltration method was able to increase the specific activity of lipase *Geobacillus thermodentrificans* by 3% (Table 1).

Due to method of salting in and salting out precipitation of ammonium sulphate take place. The solubility of

Table 1. Partial purification lipase of *Bacillus halodurans* CM1 mutant

Steps of partial purification	Total protein (mg)	Total activity (Unit)	Spesific activity (Unit/mg)	Yield (%)	Purifity (fold)
Crude enzyme	50.00	475.00	9.50	100	1.00
Ultrafiltration	5.78	60.00	10.38	12.63	1.09
UF – AS – Dialysis	0.65	9.19	14.12	1.94	1.49
U F - PEG	0.30	3.50	11.82	0.74	1.25

UF = strirred-cell ultrafiltration; AS = ammonium sulfate/$(NH_4)_2SO_4$ precipitation; PEG = polyethylene glycol.

protein will increase as the salt concentration increases (salting in), while a continuous addition of salt will result in decreased protein solubility (salting out), meaning that the protein is almost completely precipitated. Lipase of *B. halodurans* CM1 mutant from ultrafiltration (UF) was concentrated with ammonium sulphate until it reached a fraction of 20%, then continued dialysis increased specific activity from 10,38 U/mg to 14,12 U/mg. The increased activity of the enzyme, in accordance to the research of lipase from sea bacterial isolates of Pelabuhan Panjang, Bandar Lampung by Nurhasanah and Herasasi (2008), reported that concentration by ammonium sulphate increased enzyme activity from 0.21 U/mL to 3.50 U/mL.

Lipase from *B. halodurans* of CM1 mutant concentration with UF was also achieved by using polyethylene glycol (PEG). The dialysis membrane used had a pore size of 12000, while PEG measures 20000. PEG will cause molecules in enzymes smaller than 12000 to be attracted to the membrane, however it could not enter because the size is greater than 20000. Lipase of *B. halodurans* mutant CM had increased specific activity from 10,38 U/mg to 11,82 U/mg. Another study conducted by Padilha *et al*. (2012) reported that lipase activity from *Burkholderia cepacia* increased when concentrated using PEG.

SDS-page. Figure 1 suggests that protein bands appear in all samples except the crude enzyme. UF, UF-AS-dialysis and UF-PEG enzyme samples have one band each between the 30 KDa and 45 KDa markers.

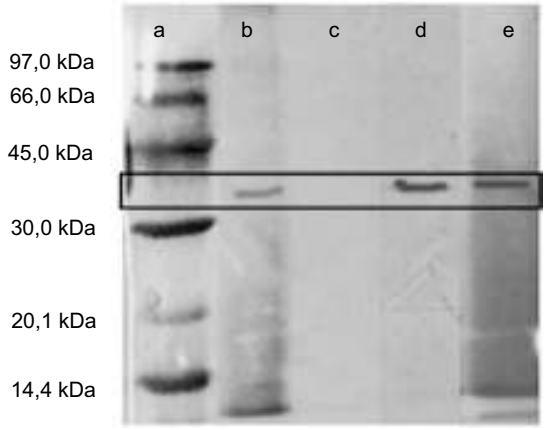

Fig. 1. SDS-Page results: a = Marker; b = UF sample; c = Crude enzyme sample; d = UF-AS-Dia-lysis sample; e = UF-PEG.

Estimation of molecular weight range lipase of 30-45 KDa, the genus *Bacillus* also has a molecular weight ranging between 19-40 KDa (Rabbani *et al*., 2015; Shah and Bhatt, 2012; Sangeetha *et al*., 2010).

Profile of pH and temperature. The optimum conditions for lipase of *B. halodurans* mutant CM1 were determined on the basis of the highest activity achieved, that being at pH 7 and temperature 50 °C, (Fig. 2-3).

The decreasing activity occurred at the basic pH after optimum pH was obtained, at pH 11 and 12, where no enzyme activity was detected, and at a high pH, where irreversible denaturation will occur. At low levels, hydrolysis occurs in unstable peptide bonds, and changes

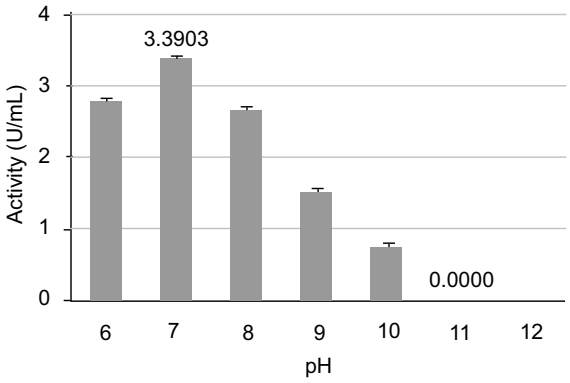

Fig. 2. The pH profile of lipase of *B. halodurans* CMl mutant, pH 7 = phosphate buffer; pH 8 & 9 = Tris-HCl buffer; pH 10, 11 and 12 = buffer glycinbe-NaOH; activity of enzyme at T = 37 °C, incubation 20 min.

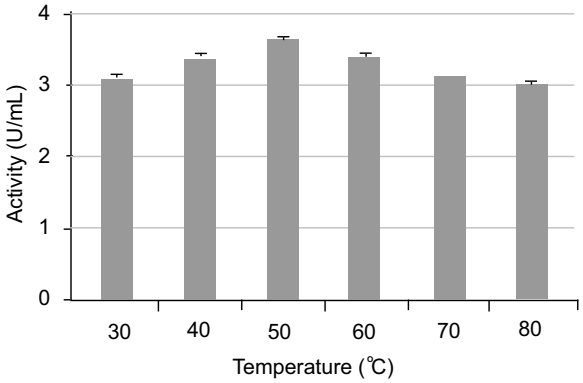

Fig. 3. The temperature profile of lipase *B. halodurans* CMl mutant, enzyme activity at = 37 °C, pH = 7, incubation 20 min.

in the enzyme activity that was affected by pH occurred due to the enzyme ionization changes.

When enzyme production, temperature controls the synthesis of enzymes at the time of transcription of mRNA and protein translation, high-temperature protein stability is established, Bora and Bora (2012). The increase in temperature to the optimum is due to the increased kinetic energy that accelerates the movement and rotation of the enzyme molecules and substrate, thus increasing the collision frequency which provides a second chance to react. Above the optimum temperature the enzyme activity decreases, possibly due to a conformational change in the protein structure so that the reactive group may experience resistance to enter the active site of the enzyme, Tunggala *et al*. (2014).

Stability of pH and lipase temperature. The lipase activity of *B. halodurans* CM1 mutant is stable at pH 7-8 (Fig. 4). In general, bacterial lipase has an optimum pH of neutral of alkaline and is stable at pH 4-11, Qamsari *et al*. (2011). Wahyuni (2016) has confirmed that lipase derived from yeast is stable at pH 7-9, the lipase of *B. coagulans* BTS-3 was stable at pH 8-10.5.

Figure 5 showed that the activity lipase of *B. halodurans* CM1 mutant is relatively stable at 30 °C and drops at 40-50 °C before becoming stable again at 60-65 °C. The lipase being stable at high temperature (> 50 °C) is most likely due to polyamines in protein structures. In addition, the amount of hydrogen bond, increased salt bridges (sulphides), hydrophobic interactions, and high thermophilic amino acid proportions also affect lipase stability against temperature, Bora and Bora (2012).

Influence of metal ion. All the metal ions tested were able to increase the lipase activity of *B. halodurans* CM1 mutant as shown in Fig. 6. Ion Ca^{2+} provided the highest activity at a concentration of 10 mM (5.20 U/mL). In addition to Ca^{2+}, Zn^{2+} ions also provide a high activity, either at concentrations of 1 mM or 10 mM (5.13 U/mL).

The enzymes require certain metal ions to increase activity by functioning as a cofactor for enzymes that can stabilize the binding to the substrate. The Ca^{2+} ions can induce changes in the conformation of enzyme

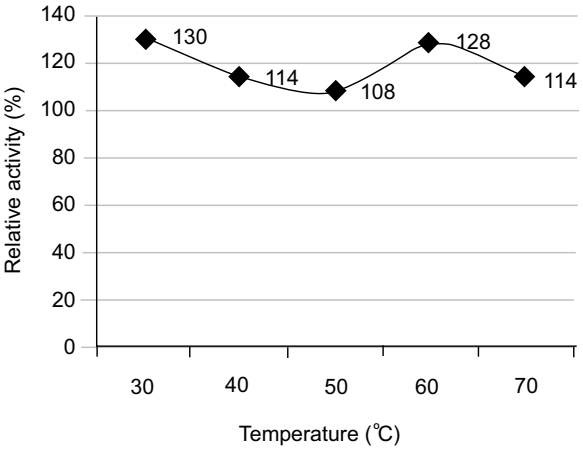

Fig. 5. Effect of temperature on lipase activity stability, incubation time 90 min, enzyme activity analysis at pH 7 and temperature 50 °C.

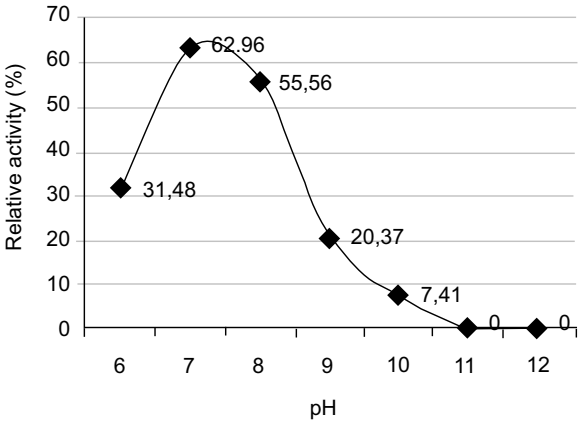

Fig. 4. Effect of pH on lipase activity stability, incubation time 90 min, analysis of enzyme activity at pH 7 and temperature 50 °C.

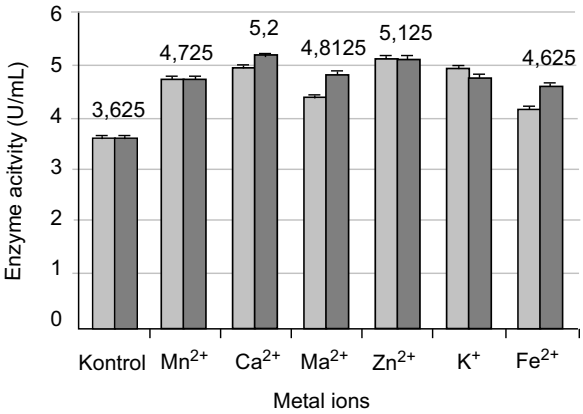

Fig. 6. Effect of addition of 1 mM and 10 mM metal ions to lipase activity.

structures to become more stable, thus increasing enzyme activity, Iqbal and Rahman (2015).

Km and Vmax. The data lipase of *B. halodurans* CM1 mutant activity test on various substrate activities were plotted on a graph, with the equation y = 0.0561 + 0.2458 and a Km value of 0.23 mg/mL and Vmax of 4.07 U/mL. The smaller the Km value, the higher the affinity for the substrate, thus indicating that a lower substrate concentration was required to reach the maximum catalytic reaction rate (Vmax), Dali *et al*. (2011). Nurhasanah (2008) obtained Km of 0.07 mg of substrate/mL and Vmax of 1.51 U/mL of lipase with bacteria isolated from seawater.

Stability of detergent and washing test. The stability test lipase of *B. halodurans* CM1 mutant is illustrated in Fig. 7. The relative activity of enzyme was decreased along with an increase in detergent concentration. Lipase mutant showed stable additions of detergent concentration 1-2% (69.64-57.14%). The activity lipase decreased by almost 93%. An addition of detergent could cause a loss of lipase activity because it alters the structure of tertiary, however, it may also maintain lipase activity and stability by inhibiting lipase agglomeration, Li *et al*. (2014).

The result of the washing test of lipase enzyme on the cloth was found to lose 8.40% of oil content, where the control was only able to remove 2.40%. The washing test aims to determine the ability of lipase in hydrolyzing oil on the cloth, therefore the addition of *B. halodurans* CM1 mutant affects and the oil hydrolysis capability of the cloth even though it was still very low. The amount of oil lost from the cloth in the presence of enzyme addition was still higher than without the addition of the enzyme (control).

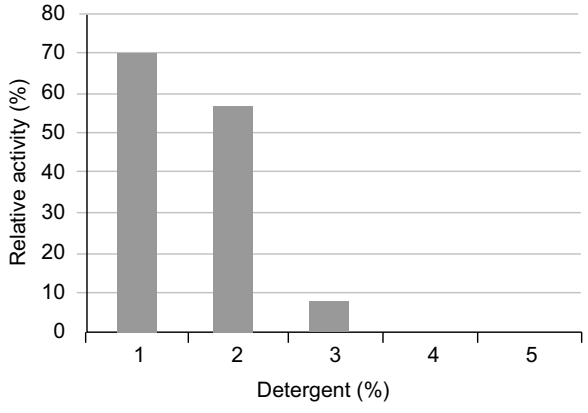

Fig. 7. Relative activity lipase of *B. halodurans* CMl mutant against detergent with 45 min incubation time.

Conclusion

It can be concluded that lipase mutant *B. halodurans* CM1 activity can be increased by purification, where the SDS page can estimate the molecular weight. The optimum condition of lipase enzyme was achieved at pH 7 and temperature 50 °C, whereas stability was observed at pH 7-8 and temperature 30-70 °C. The highest Ca^{2+} ions increased lipase activity and was stable with the addition of detergent concentration of 1-2%. The washing test concluded that 8.40% is the enzyme capable of hydrolysing oil on cloth.

Aknowledment

This research was supported by the Center for Bio-industrial Technology (PTB), Agency for the Assessment and Application of Technology (BPPT), which provided funding and laboratory facilities to conduct this study.

Conflict of Interest. The authors declare no conflict of interest.

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Pak. j. sci. ind. res. Ser. B: biol. sci. 2020 **63B**(3) 94-108

Review

A Review of Taxonomic Perspective of Diversity in Gymnosperms of Kashmir Himalaya

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(received May 28, 2018; revised January 11, 2019; accepted January 21, 2019)

Abstract. Varied floristic works relevant to Kashmir Himalaya were thoroughly examined to compile taxonomic contributions of various taxonomists *viz*. a *viz*. diversity in gymnosperms of this region. Extensive field surveys and standard taxonomic methods were used to locate, collect, identify and prepare an updated list of the target group. A total of 25 species of gymnosperms belonging to 13 genera in 6 families and 4 orders have been documented from the Kashmir Himalaya. Wild gymnosperms are represented by 11 species with conifers forming the most dominant group. Among families, Pinaceae is highest represented with 6 species, while Taxaceae is least represented. Cultivated gymnosperms exceed wild growing species, and Cupressaceae is most dominant with 9 species whereas Ginkgoaceae is least represented. Out 25 species 19 (7 wild + 12 cultivated) are trees, 5 (3 wild + 2 cultivated) are shrubs and only 1 is sub-shrub.

Keywords: floristic diversity, gymnosperm, Kashmir Himalaya, cultivation

Introduction

Gymnosperms are distributed throughout the world with about 1079 species in 12 families and approx. 83 genera (Christenhusz and Byng, 2016). The three 'non-conifer' groups comprise about 337 species of cycads in 10 genera, one extant ginkgophyte, and 111 species of gnetophytes in three genera (Christenhusz and Byng, 2016). Farjon (2010) has reported about 615 species of conifers in 70 accepted genera, however according to Christenhusz and Byng (2016) their number is 629 species in 69 genera and 06 families. The genus *Juniperus* L. represented by 75 species is considered to be one of the most diverse genera of gymnosperms distributed from sea level to above tree-line zone (Lakusic and Lakusic, 2011).

India is abode to about 101 species, 9 varieties and 1 form of gymnosperms belonging to 33 genera under 10 families with about 44 species in wild (Srivastava, 2006). A total of 63 species of gymnosperm are reported from western Himalaya (Tewari *et al*., 2010). Although much less in numbers gymnosperms still constitute dominant element of forests of world in temperate areas of both the northern and southern Hemispheres. They occur in all continents except Antarctica and form outstanding feature of landscape of the Himalaya. The existing gymnosperms belong to six orders: Cycadales, Ginkgoales, Coniferales, Gnetales, Welwitschiales and Ephedrales.

Forests are verily the green gold of the State of Jammu and Kashmir play a vital role in the maintenance of natural balance which is of paramount importance in a mountainous region like ours. Despite their immense ecological and socio-economic values the taxonomy of gymnosperms has been neglected in the Indian sub-continent, especially in the Kashmir Himalaya. Even though Hooker (1888) gave the first taxonomic treatment of gymnosperms of India, but he cited very little material. Since then some local researchers (Dar and Christensan, 2003; Javeid, 1979, 1970; Singh and Kachroo, 1976; Dhar, 1966) attempted to deal with gymnosperm floristics in this region, but the taxonomic intricacy regarding this group in the Kashmir Himalaya still persists.

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It is for these reasons that present investigation was carried out to compile contributions of various taxonomists *viz*. a *viz*. diversity in gymnosperms of this region, validate and augment it with extensive field studies so as to present an updated inventory of gymnosperm species in the Kashmir Himalaya.

Study area. Kashmir Himalaya being located at the bio-geographically pivotal position, represents a unique biotic province in the northwestern extreme of the Himalayan range. The region lies between coordinates 32° 17' to 37° 20' north latitude and 73° 25' to 80° 30' east longitude spreading over an area of about 2, 22, 235 km2 (Hussain, 2001). It comprises mostly of rugged terrain, except for small plains of Jammu and vale of Kashmir, and encompasses four categories of bio-geographic biomes: Tundra, Alpine, Temperate and Subtropical (Rodgers and Panwar, 1988). The valley of Kashmir is an oval plain that lies between 32° 20' to 34° 50' north latitude and 73° 55' to 75° 35' east longitude covering an area of about 16,000 sq. km. It is formed by a girdling chain of the Himalayan mountains, namely the Pir Panjal range in the south and the great Himalayan range all along the southeast through northeast to the west. The entire territories of the Kashmir valley form two distinct topographic divisions, the mountain ranges and the valley proper. It extends roughly 187 km in length and about 116 km in breadth along the latitudes of Srinagar. On an average, the climate of valley is temperate with bixeric regimes, having two dry spells in June and September, and high precipitation during the winter season.

Materials and Methods

After thorough study of the available herbarium specimens in Kashmir University Herbarium (KASH) and comprehensive literature evaluation from varied sources, different forest habitats in the Kashmir Himalaya were explored for collection of specimens of different gymnosperm species. They were assigned a specific field number and on spot diagnostic characters were noted in the field book. In case of *Abies*, *Picea* and *Cedrus*, where all the leaves (needles) fell down the twigs a few days after direct pressing, the method proposed by page (1979) proved effective. Female cones of *Cedrus* and *Abies*, where bract and ovuliferous scales fall, while still on the tree, were collected just prior to dismembering stage and kept air tight in small polythene bags so as to keep them intact. The specimens were identified using the available literature on floristics of this region. Data pertaining to enumeration and distribution of taxa along with altitude (wherever available) has been tabulated.

Taxonomic appraisal. The preliminary work on taxonomy of gymnosperms in India actually commenced from Drury, who in (1869) reported a few gymnosperms from India. Hooker's work (1888) is however, considered to be the first solid step in this direction. Treated the gymnosperms as Gymnospermae and reported 30 species in 16 genera belonging to 3 families (natural orders) from the British India. Out of these, 11 species spread over 5 genera and 2 families have been cited from the Kashmir Himalaya (Table 1). Hooker also recognized six tribes in order Coniferae on the basis of position of ovule, as follows:

A. Ovule erect:	I. Cupressinae II. Taxodiaceae III. Taxeae
B. Ovule reversed:	I. Podocarpeae II. Araucarieae III. Abietineae

Hooker (1888) and Gamble (1902), each recognized four Juniper species from India. These are as follows:

Juniperus macropoda	- Inner drier regions of the Himalaya from Afghanistan to Nepal (1500-4500m).
J. communis	- Western Himalaya from Nepal westwards (2000-5000m).
J. recurva	- Himalaya from Afghanistan to Bhutan (3000-4500m).
J. pseudosabina	- Himalaya from Afghanistan to Bhutan (3000-4500m).

Brandis (1906) reported 47 species of gymnosperms belonging to 12 genera and 3 families from the British Indian Empire. Out of these, 14 species belonging to 8 genera within 2 families were cited from Kashmir Himalaya (Table 2). Lambert (1933) in his list of trees and shrubs for Kashmir and Jammu forest circles reported 13 species of gymnosperms belonging to 7 genera (Table 3).

Raizada and Sahni (1960) have reported 42 species of gymnosperms belonging to 14 genera in 7 families from the Indian sub-continent. Out of these, 13 species belonging to 7 genera in 3 families have been cited

from the Kashmir Himalaya (Table 4). Raizada and Sahni (1960) have documented 6 species of Junipers from the Himalaya: two species with scaly leaves in the adult stage, *viz*. *Juniperus wallichiana* and *J. macropoda* and other 4 species with acicular adult foliage, namely *J. coxii*, *J. recurva*, *J. squamata* and *J. communis*.

Wali and Tiko (1964) have reported 7 species belonging to 6 genera in a single family Coniferae from the Lolab valley in Kashmir. These are as follows:

Coniferae:

Juniperus	-	*J. communis*, *J. recurva*
Taxus	-	*T. baccata*
Pinus	-	*P. wallichiana*
Cedrus	-	*C. deodara*
Picea	-	*P. smithiana*
Abies	-	*A. pindrow*

Table 1. Gymnosperm taxa reported from British India (Hooker, 1888)

Name of family	Genus Number	Genus Name	Species Number	Species Name	Taxa cited from Kashmir Himalaya
Gnetaceae	2	Ephedra	3	*E. vulgaris* *E. pachyclada* *E. peduncularis*	*E. vulgaris* (Western Tibet). *E. pachyclada* (Western Himalaya, Western Tibet).
		Gnetum	6	*G. gnemone* *G. neglectum* *G. macrostachyum* *G. scandens* *G. funiculare* *G. macropodum*	--
Coniferae	13	Cupressus	3	*C. torulosa* *C. sempervirens* *C. funebris*	*C. sempervirens* (Northwest India). *C. torulosa* (Western Himalaya).
		Juniperus	4	*J. communis* *J. pseudosabina* *J. recurva* *J. macropoda*	*J. pseudo-sabina* (Kashmir to Bhutan). *J. communis* (Western Himalaya, Kumaon westwards). *J. macropoda* (Western Tibet, 5000 - 14000f).
		Cephalotaxus		*C. manii* *C. griffithi*	--
		Taxus		*T. baccata*	*T. baccata* (Temperate Himalaya).
		Dacrydium Podocarpus		*D. elatum* *P. latifolius* *P. neriifolius* *P. cupressine*	--
		Agathis		*A. loranthifolia*	
		Pinus	5	*P. excelsa* *P. longifolia* *P. khasya* *P. gerardiana* *P. merkusii*	*P. excelsa* (Temperate Himalaya, 6000-12000f). *P. longifolia* (Indus-Bhutan, 1500-6000f). *P. gerardiana* (Dry interior valleys of North-west Himalaya, 5000-12000f).
		Cedrus	1	*C. libani*	--
		Picea	1	*P. morinda*	
		Tsuga	1	*T. brunoniana*	
		Abies	1	*A. webbiana* var. *webbiana*	
		Larix	1	*L. griffithii*	
Cycadaceae	1	Cycas	5	*C. circinalis* *C. rumphii* *C. pectinata* *C. siamensis* *C. beddomei*	-- --
Total = 3	16	--	39	--	11

Dhar (1966) while dealing with distribution of Pinaceae in India has cited 14 indigenous species of gymnosperms belonging to 12 genera and 6 families of order Coniferales. Out of these, 7 species in 4 genera of family Pinaceae have been cited from the Kashmir Himalaya (Table 5). Dallimore and Jackson (1966) have reported only 5 species of Junipers from Himalaya, *viz*. *J. communis*, *J. macropoda*, *J. recurva*, *J. squamata* and *J. wallichiana* (=*J. pseudosabina*). It is worth mentioning here that *J. coxii has* been treated as a variety of *J. recurva*.

Table 2. Gymnosperm taxa reported from Kashmir Himalaya (Brandis, 1906)

Name of family	Genus		Species		Distribution in Kashmir Himalaya
	Number	Name	Number	Name	
Gnetaceae	1	Ephedra	3	*E. gerardiana (E. vulgaris)* *E. nebrodensis* *E. intermedia*	Himalaya: Kuram Valley, Yarkand, Tibet. Baltistan. Northern Himalaya: Gilgit, Zanskar, Upper Chenab.
Coniferae	13	Pinus	2	*P. excelsa* *P. gerardiana*	Bhutan to Afghanistan. Northwest Himalaya: Gilgit, Indus between Astor and Iskardo, Upper Chenab.
		Cedrus	1	*C. deodara (C. libani var. deodara)*	Northwest Himalaya (4000-10000f).
		Abies	2	*A. pindrow* *A. webbiana*	Kurram valley, eastwards to Nepal. Northwest Himalaya (10000-14000f).
		Picea	1	*P. morinda (Abies smithiana)*	Himalaya: Common from Kashmir to Garhwal and Gilgit.
		Cupressus	1	*C. sempervirens*	Planted in gardens of Northwest India.
		Juniperus	3	*J. communis* *J. recurva* *J. macropoda (J. excelsa)*	Northwest Himalaya (5400-14000f). Kashmir to Bhutan (7500-12000f). Inner arid ranges and valleys of Northwest Himalaya.
		Taxus	1	*T. baccata subsp. wallichiana*	India.

Table 3. Gymnosperm taxa reported from Kashmir and Jammu forest circles (Lambert, 1933)

Name of genus	Species		Distribution in Kashmir Himalaya
	Number	Name	
Ephedra	2	*E. gerardiana* *E. intermedia*	Gurez, Jhelum Valley. Kishanganaga, Sind Valley. Kashmir, Baltistan, Chenab valley, Jammu, Mirpur, Muzaffarabard, Udhampur.
Pinus	3	*P. longifolia* *P. gerardiana* *P. excelsa*	Baltistan, Chenab valley, Jammu, Kamraj, Mirpur, Muzaffarabard, Udhampur. Mainder, Kashmir. Jhelum Valley, Gurez, Kamraj, Kishanganaga, Mainder, Muzaffarabad, Sind Valley, Udhampur.
Picea	1	*P. smithiana*	Gurez, Jhelum Valley. Kishanganaga, Kashmir, Mainder, Muzaffarabard, Sind Valley, Udhampur.
Abies	1	*A. pindrow*	Gurez, Jhelum Valley. Kamraj, Kishanganaga, Kashmir, Sind Valley, Udhampur.
Cedrus	1	*C. deodara*	Gurez, Jhelum Valley, Kamraj, Kishanganaga, Kashmir, Mainder, Muzaffarabard, Sind Valley, Pir Panjal, Udhampur.
Juniperus	4	*J. communis* *J. squamata* *J. macropoda* *J. pseudosabina*	Gurez, Jhelum Valley. Kashmir, Mainder, Sind Valley. Gurez, Jhelum Valley. Kishanganaga, Kashmir. Mainder, Sind Valley, Gurez, Kishanganaga, Sind Valley, Kashmir.
Taxus	1	*T. baccata*	Gurez, Jhelum Valley. Kamraj, Kishanganaga, Kashmir, Mainder, Muzaffarabard, Sind Valley, Udhampur.

Gaussen (1968) has reported 7 species of Junipers from the Himalaya, as follows:

Name of species		Distribution
J. pseudosabina	-	Throughout the Himalaya.
J. wallichiana	-	Indus to Bhutan (2700-4500m).
J. religiosa (*J. excelsa*)	-	Drier ranges of Northwest Himalaya from Kashmir to Nepal (1500-3000m).
J. macropoda (4000m).	-	Afghanistan to western Nepal
J. squamata	-	Himalaya (3,000-5,000m) in Alpine zone.
J. recurva	-	Eastern Himalaya in temperate or sub-alpine zone in Sikkim and Bhutan (3,000-4000m); Afghanistan, Kashmir, Tibet.

Javeid (1970) has described 11 species of gymnosperms belonging to 10 genera and 5 families from Srinagar Kashmir (Table 6). Stewart (1972) treated gymnosperms as class Gymnospermae and cited 35 species in 14 genera belonging to 5 orders of them from Pakistan and Kashmir. Out of these, 17 species in 7 genera have been recognized from the Kashmir Himalaya (Table 7). Koul and Sarin (1974) studied the vertical distribution of plant communities between 1,000 and 3900m altitudes in Bhaderwah hills (western Himalaya) and found a well marked zonation of vegetation from lower to upper elevation. The lower zone (1,600-1,900m) comprises *Quericus incana*, *Q. floribunda* and *Pinus wallichiana*; the intermediate slopes (2,000-2,600m) consists of almost pure stands of *Cedrus deodara*, while locations from 2,000-3,700m are dominated by *Abies pindrow* forests. The oaks were restricted to drier situations and conifers to moist slopes. Dhar (1975) has reported a healthy, well-grown fertile specimen of a prodigious

Table 4. Gymnosperm taxa reported from Kashmir Himalaya (Raizada and Sahni, 1960)

Name of family	Genus		Species		Distribution
	Number	Name	Number	Name	
Pinaceae	4	Abies	2	*A. pindrow*	Afghanistan to Nepal (2300-3300m).
				A. spectabilis	West Pakistan to Bhutan (3300-4000m).
		Cedrus	1	*C. deodara*	Afghanistan to Garhwal.
		Picea	1	*P. smithiana*	Afghanistan to Kumaon (2150-3200m).
		Pinus	5	*P. gerardiana*	From Bashahar westwards to Kashmir.
				P. roxburghii	Afghanistan to Bhutan (450-2300m).
				P. wallichiana	All along Himalaya 1800-3700m).
Taxaceae	1	Taxus	1	*T. baccata*	All along Himalaya 1800-3700m).
Cupressaceae	2	Cupressus	1	*C. torulosa*	West Pakistan and Kashmir.
		Juniperus	4	*J. communis*	Afghanistan to Kumaon (1700-4300m).
				J. macropoda	Kashmir, (1500- 4300m).
				J. recurva	All along Himalaya from West Pakistan to Bhutan (2300-4600m).
				J. wallichiana	Indus Nepal (3300-4200m).

Table 5. Gymnosperm taxa reported from Kashmir Himalaya (Dhar, 1966)

Name of family	Genus		Species		Distribution
	Number	Name	Number	Name	
Pinaceae	4	Pinus	3	*P. roxburghii*	Kashmir to Bhutan.
				P. wallichiana	Northwestern Himalaya.
				P. gerardiana	Bashahar to Kashmir.
		Cedrus	1	*C. deodara*	North-west Himalaya from Garhwal to Kashmir.
		Picea	1	*P. smithiana*	Western Himalaya from Kashmir to Kumaon.
		Abies	2	*A. pindrow*	Western Himalaya from Kashmir to Nepal. Western Himalaya.
				A. spectabilis	

conifer- *Sequoiadendron giganteum* growing in the Yarikha Drug Farm, Tangmarg Kashmir.

Mehra (1975) using taxonomic and anatomical tools has tried to resolve the Abies and Juniperus complexes existing in the Himalaya. According to author 4 types of firs are met within the western Himalaya *viz*. (1) high-level *Abies spectabilis* (2) high-level hybrid (3) low-level hybrid and (4) low-level *A. pindrow*. Regarding *Juniperus* complex, the author has concluded that in all there are 9 species of Junipers in the Himalaya, of these 5 are trees and 4 shrubs. *Juniperus pseudosabina* is a shrub occurring all-along the Himalayan range from west to east. Out of the other 8 species, 4 are met within the western Himalaya and an equal number in the eastern Himalaya. The western Himalayan Junipers are: *J. communis*, *J. squamata* (both shrubs), *J. macropoda* and *J. excelsa var. Farreana* (both trees).

Singh and Kachroo (1976) have reported 6 species of gymnosperms in 5 genera belonging to 2 families from Srinagar. These are as follows:

Pinaceae:

Abies - *A. pindrow*
Juniperus - *J. communis*, *J. recurva*
Picea - *P. smithiana*
Pinus - *P.griffithii*

Taxaceae:

Taxus - *T. wallichiana*

Based on extensive survey of *Juniperus* populations in various parts of the Himalaya and the details of morphological and anatomical characters, Jain (1976) has reported 8 distinct taxa of Juniper from the entire Himalayan range. In the eastern Himalaya 5 taxa are present, 3 (*J. wallichiana*, *J. recurva* and *J. fargesii*) are trees and restricted to the eastern Himalaya, while the other 2 also extend to the western Himalaya. The west Himalayan taxa are also 5 in number: *J. macropoda* and *J. excelsa* being trees, whereas *J. communis* ssp. *Nana*, *J. squamata* and *J. pseudo-sabina* are shrubs.

Table 6. Gymnosperm taxa reported from Kashmir Himalaya (Javeid, 1970)

Name of family	Genus		Species	
	Number	Name	Number	Name
Ginkgoaceae	1	Ginkgo	1	*G. biloba*
Pinaceae	4	Pinus	2	*P. wallichiana* *P. halepensis*
		Cedrus	1	*C. deodara*
		Picea	1	*P. smithiana*
		Abies	2	*A. pindrow*
Cupressaceae	3	Thuja	1	*T. orientalis*
		Cupressus	1	*C. arizonica*
		Juniperus	1	*J. communis*
Taxaceae	1	Taxus	1	*T. wallichiana*
Ephedraceae	1	Ephedra	1	*E. gerardiana*

Dhar (1978) has reported 8 species of gymnosperms from the Kashmir Himalaya. Among these 6 species *viz*. *Pinus wallichiana*, *P. excelsa*, *Cedrus deodara*, *Picea smithiana*, *Abies pindrow*, *Taxus baccata* are distributed along temperate and subalpine region, while the rest 2 species i.e., *Juniperus communis* and *Juniperus recurva* are distributed along subalpine to alpine region. Javeid (1979) has reported 19 species of gymnosperms belonging to 19 genera in 4 orders and 5 families under a single class Gymnospermae from the Kashmir Himalaya. Out of these, 3 species are grown as ornamentals, while the rest occur as wild (Table 8). Lancaster (1980) has reported 6 species of gymnosperms from Kashmir *viz*. *Abies pindrow*, *Pinus wallichiana*, *Taxus wallichiana*, *Picea smithiana*, *Juniperus communis* spp. *Montana* (*nana*), and *Juniperus macropoda*. The specimens of these taxa have been collected from Lidder valley, Gulmarg and Lashpatri, Sonamarg and Vishensar in Sind valley regions of Kashmir. Polunin and Stainton (1984) have reported 22 species of gymnosperms belonging to 11 genera and 5 families from the entire Himalayan region (Table 9). Bhat (1984) has reported 5 species of gymnosperms (*Pinus wallichiana*, *Picea smithiana*, *Cedrus deodara*, *Abies pindrow* and *Juniperus communis*) from Gulmarg area. These are distributed along forest slopes from Tangmarg to Khillanmarg.

Mehra (1988) has described 38 species of gymnosperms belonging to 13 genera from India. Out of these, 29 species falling into 11 genera have been treated under conifers and the rest 9 species belonging to 2 genera have been treated under Gnetophytes (Table 10). Conifers are mostly confined to Himalaya especially in north-west and western regions. Pinaceae among conifers has the maximum representation with 14 species in 2 genera. Cephalotaxaceae, Podocarpaceae and Taxaceae are each represented by a single genus with 2 species each in the first two and only one species in last one. Taxodiaceae and Araucariaceae are not represented in wild. Out of total, 15 species of gymnosperms belonging to 7 genera have been reported from the Kashmir Himalaya. Among these, 12 species belonging to 6 genera fall under conifers and the rest 3 species belonging to 1 genus fall under Gnetophytes.

Sahni (1990) has reported 61 species of gymnosperms belonging to 16 genera in 8 families from the Indian sub-continent. Out of these, 54 species belonging to 15 genera in 8 families have been cited from India; these includes 23 species belonging to 8 genera in 4 families reported from the Kashmir Himalaya (Table 11).

Singh and Kachroo (1994) have reported 7 species of gymnosperms belonging to 6 genera in 3 families from the Pir Panjal range. These are as follows:

Pinaceae:

Abies - *A. pindrow*
Cedrus - *C. deodara*
Pinus - *P. wallichiana*

Cupressaceae:

Juniperus - *J. communis, J. recurva*
Picea - *P. smithiana*

Taxaceae:

Taxus - *T. baccata*

Table 7. Gymnosperm taxa reported from Pakistan and Kashmir (Stewart, 1972)

Name of order	Family		Genus		Species		Taxa cited from Kashmir Himalaya
	Number	Name	Number	Name	Number	Name	
Cycadales	-	-	2	Cycas	2	*C. revoluta*	
						C. rumphii	-
				Zamia	1	*Z. integrifolia*	-
Ginkgoales	-	-	1	Ginkgo	1	*G. biloba*	-
Ephedrales	-	-	1	Ephedra	9	*E. ciliata*	*E. gerardiana*
						E. gerardiana	(Kashmir, 5500-17000f).
						E. intermedia	*E. intermedia* var. tibetica (Kashmir).
						var. *gluaca*	*E. pachyclada* (W. Tibet, probably Ladakh).
						E. intermedia	*E. procera* (Kishtwar).
						var. *tibetica*	*E. przewalskii* (Nanga Parbat).
						E. pachyclada	*E. regeliana* (Ladakh).
						E. procera	
						E. przewalskii	
						E. regeliana	
						E. sarcocarpa	
						E. monosperma.	
Coniferales	4	Pinaceae	4	Abies	2	*A. pindrow*	*A. pindrow*
						A. spectabilis	(Afghanistan to Kumaon, 7-10000f).
						A. spectabilis	(Afghanistan to Bhutan, 8-13000f).
				Cedrus	1	*C. deodara*	*C. deodara* (Kashmir, 4-10000f).
				Picea	1	*P. smithiana*	*P. smithiana* (Kashmir, 6-11000f).
				Pinus	4	*P. gerardiana*	*P. gerardiana* (Kishtwar).
						P. roxburghii	*P. roxburghii* (Afghanistan to Bhutan 1500-6000f).
						P. wallichiana	
						P. halepensis	
		Taxodiaceae	1	Taxodium	1	*T. mucronatum*	-
		Araucariaceae	1	Araucaria	1	*A. cookie*	-
		Cupressaceae	3	Cupressus	5	*C. funebris*	
						C. lusitanica	
						C. macrocarpa	
						C. sempervirens	
						C. torulosa	-
				Juniperus	5	*J. communis*	*J. communis* var. saxatilis (Dras, Ladakh,
						var. *saxatilis*	Kashmir 8-14000f).
						J. excelsa	*J. squamata* (Kashmir 8-14000f).
						J. squamata	*J. turkistanica* (Nanga Parbar).
						J. turkistanica	*J. wallichiana* (Kishtwar, chiefly east of Kashmir).
						J. wallichiana	
				Thuja	1	*T. orientalis*	-
Taxales	-	-	1	Taxus	1	*T. wallichiana*	*T. wallichiana* (Poonch, Kashmir 6-11000f).
Total = 5	4	-	14	-	35	-	17

Ara *et al*. (1995) have reported 295 indigenous and exotic species in 12 genera under 60 families from the Kashmir Valley. Out of these, 18 species in 11 genera under 5 families belong to gymnosperms; 15 species in 10 genera under 5 families being trees and the remaining 3 species in one genus being shrubs. Further out of these gymnosperms, 9 species in 6 genera and 4 families are exotic, while the remaining 9 species in 7 genera and 3 families are indigenous to Kashmir (Table 12). Dar *et al*. (2002) have reported 26 species (both wild as well as cultivated) of gymnosperms belonging to 12 genera and 6 families from the Kashmir valley. These are as follows:

Wild growing gymnosperms in Kashmir

Cupressaceae:

Cupressus - *C. torulosa*
Juniperus - *J. communis, J. semiglobosa, J. squamata*

Ephedraceae:

Ephedra - *E. gerardiana*

Pinaceae:

Pinus - *P. wallichiana, P. roxburghii*
Abies - *A. pindrow, A. spectabilis*
Cedrus - *C. deodara*
Picea - *P. smithiana*

Taxaceae:

Taxus - *T. wallichiana*

Exotic gymnosperms grown as ornamentals in Kashmir

Cupressaceae:

Cupressus - *C.arizonica, C. cashmeriana, C. corneyana, C. glabra, C. guadalupensis, C. sempervirens*
Juniperus - *J. chinensis*
Thuja - *T. orientalis*

Ginkgoaceae:

Ginkgo - *G. biloba*

Pinaceae:

Pinus - *P. canariensis, P. halepensis, P. radiata*

Taxodiaceae:

Cyptomeria - *C. japonica*
Sequoiadendron - *S. giganteum*

Khan (2002) has reported 5 species of gymnosperms in 5 genera belonging to 3 families from Bijhama (Uri) and its adjacent areas in Kashmir as below:

Pinaceae:

Pinus - *P. wallichiana*
Cedrus - *C. deodara*
Abies - *A. pindrow*

Taxaceae:

Taxus - *T. baccata*

Ephedraceae:

Ephedra - *E. gerardiana*

Table 8. Gymnosperm taxa reported from Kashmir Himalaya (Javeid, 1979)

Name of order	Family		Genus		Species		Distribution in the Kashmir Himalaya
	Number	Name	Number	Name	Number	Name	
Ginkgoales	-	-	1	Ginkgo	1	*G. biloba*	Lanlmandi garden (cultivated).
Ephedrales	-	-	1	Ephedra	3	*E. gerardiana* *E. vulgaris* *E. intermedia*	Gurez, Jhelum and Sind valley. Srinagar, Ldakh, Zanskar. Gurez.
Coniferales	2	Pinaceae	4	Pinus	4	*P. wallichiana* *P. roxburghii* *P. gerardiana* *P. halepensis*	Gurez, Jhelum, Lolab and Sind valley, Gulmarg, Pahalgam. Chenab Valley, Shankarachaya Hill (planted). Chenab Valley. Shankaracharya (planted).
				Picea	1	*P. smithiana*	Gurez, Jhelum, Lolab and Sind valley.
				Abies	1	*A. pindrow* *A. spectabilis*	Gurez, Jhelum, Lolab and Sind valley. Common in eastern Himalaya and also reported from western Himalaya.
				Cedrus	1	*C. deodara*	Gurez, Jhelum, Lolab and Sind valley.
		Cupressaceae	2	Cupressus	1	*C. torulosa*	Srinagar (planted).
				Juniperus	3	*J. communis* *J. squamata* *J. wallichiana*	Gurez, Drass, Jhelum, Lolab and Sind valley. Common and often with J. communis above the tree line. Keran, Kishtwar.
				Thuja	1	*T. orientalis*	Kashmir Valley (planted).
Taxales	1	Taxaceae	1	Taxus	2	*T. wallichiana* *T. baccata* subsp. *wallichiana*	Gurez, Jhelum, Lolab and Sind valley.

Table 9. Gymnospermous taxa reported from Kashmir Himalaya (Polunin and Stainton, 1984)

Name of family	Genus		Species		Distribution
	Number	Name	Number	Name	
Ephedraceae	1	Ephedra	1	*E. gerardiana*	Afghanistan to Bhutan.
Pinaceae	6	Pinus	3	*P. wallichiana*	Afghanistan to South Tibet.
				P. roxburghii	Afghanistan to Bhutan.
				P. gerardiana	Afghanistan to Uttar Pradesh.
		Cedrus	1	*C. deodara*	Afghanistan to West Nepal.
		Picea	2	*P. smithiana*	-
				P. spinulosa	Afghanistan to Nepal.
		Tsuga	1	*T. dumosa*	-
		Abies	3	*A. pindrow*	Afghanistan to West Nepal.
				A. spectabilis	Afghanistan to Bhutan.
				A. densa	-
		Larix	2	*L. griffithiana*	-
				L. himalaica	- Tibet.
Taxodiaceae	1	Cryptomeria	1	*C. japonica*	-
Cupressaceae	2	Cupressus	2	*C. arizonica*	-
				C. corneyana	-
		Juniperus	5	*J. communis*	Afghanistan to Nepal.
				J. recurva	Pakistan to South-west China.
				J. squamata	Pakistan to South-west China.
				J. indica	Pakistan to South-west China
				J. macropoda	Pakistan to Uttar Pradesh
Taxaceae	1	Taxus	1	*T. wallichiana* subsp. wallichiana	Afghanistan to Southwest China

Table 10. Gymnospermous taxa reported from Kashmir Himalaya (Mehra, 1988)

Name of order	Family		Genus		Species		Chromosome no.	Distribution
	Number	Name	Number	Name	Number	Name		
Coniferales	3	Pinaceae	4	Pinus	3	*P. gerardiana*	2n = 24	Inner ranges of Northwest Himalaya (1100-3300m).
						P. roxburghii	2n = 24 n = 12	Northwest Himalaya Bhutan to Afghanistan (500-2500m). Northwest temperate Himalayan
						P. griffithii	2n = 24 n = 12	forests (200-3000m).
				Cedrus	1	*C. deodara*	2n = 24 n = 12	Throughout western Himalaya (1300- 3200m), extending from Afghanistan to Garwhal.
				Picea	1	*P. smithiana*	2n = 24	Western Himalaya, Nepal to Afghanistan.
				Abies	2	*A. pindrow*	2n = 24	Western Himalaya, Nepal to Afghanistan
						A. spectabilis	n = 12	(2400-2700m). Inner west Himalayan ranges at higher elevations than that of A. pindrow.
		Cupressaceae	1	Juniperus	4	*J. macropoda*	2n = 22	Western Himalaya, Sonamarg.
						J. communis	2n = 22	Western Himalaya, Baltal.
						J. pseudo-sabina	2n = 22	Western Himalaya, from Kashmir (Baltal) to
						J. squamata		Kumoan. Western Himalaya.
		Taxaceae	1	Taxus	1	*T. wallichiana*	n = 12	Himalaya (2000-3000m), Afghanistan to Bhutan.
Gnetophytes	1	Ephedraceae	1	Ephedra	3	*E. intermedia*	n = 14	Kashmir.
						var. tibetica	2n = 28	
						E. gerardiana	n = 14	Kashmir.
						var. wallichii	n = 14	
						E. saxatilis	2n = 22	Zanaskar, South Tibet.

Khanday (2002) has reported 3 species of gymnosperms belonging to 3 genera and 2 families from Lower-Mundah and its adjacent area in Kashmir as below:

Pinaceae:
Abies - *A. pindrow*
Pinus - *P. wallichiana*

Taxaceae:
Taxus - *T. wallichiana*

Khuroo (2003) has documented 5 species of gymnosperms belonging to 5 genera in 2 families from Langate (Kupwara) as below:

Pinaceae:
Pinus - *P. wallichiana*
Cedrus - *C. deodara*
Abies - *A. pindrow*
Picea - *P. smithiana*

Taxaceae:
Taxus - *T. wallichiana*

Dar (2004) has cited a total of 20 species of gymnosperms belonging to 12 genera in 6 families from the Kashmir valley. Of these, 9 species in 7 genera ad 5 families occur in cultivation only (Table 13). The conifers, with 16 species in 9 genera and 3 families form the most dominant group. Among families, Pinaceae with 7 species in 4 genera is highest represented, while as Ginkgoaceae with 1 species is least represented.

Dar and Dar (2005) described gymnosperm specie *viz*. *Taxodium distichum* from the Kashmir Himalaya. Dar and Dar (2006) reported 16 species spread over 9 genera in 3 families of conifers from Kashmir Himalaya. They stated that family Pinaceae is dominant with 7 species and Taxodiaceae is represented by 2 species only. Further, 7 species in 5 genera have been reported to exist in cultivation. Dar and Dar (2011) updated information on *Sequoiadendron giganteum* growing in Tangmarg area of Kashmir.

Singh *et al*. (2018) documented *Juniperus chinensis* from Gurez valley in the innermost northern part of Kashmir Himalaya. Their findings suggest an extension of known geographic distribution of this species from central and southeast Asia to south Asia hence strongly supporting a relationship between northern Himalayan range of India and southern hill ranges of China.

Table 11. Gymnosperm taxa reported from Kashmir Himalaya (Sahni, 1990)

Name of family	Genus		Species		Distribution
	Number	Name	Number	Name	
Taxaceae	1	Taxus	1	*T. baccata*	Himalaya: Pakistan to Arunachal Pradesh (1800-3700m).
Pinaceae	4	Pinus	3	*P. wallichiana*	Himalaya: Pakistan to Arunachal Pradesh (1800-3700m).
				P. gerardiana	Bashahar westwards to Kashmir Chitral.
				P. roxburghii	Himalaya: Pakistan to Arunachal Pradesh (450-2300m).
		Cedrus	1	*C. deodara*	Afghanistan, Kashmir.
		Picea	1	*P. smithiana*	Western Himalaya from Afghanistan to Kumaon (2150-3200m).
		Abies	2	*A. pindrow*	Western Himalaya to Nepal (2300-3300m).
				A. spectabilis	Himalaya: Pakistan to Arunachal Pradesh, Tibet, commonly at (3300-4000m).
Cupressaceae	2	Juniperus	6	*J. polycarpos*	Kashmir, western Tibet (2500-4300m).
				J. recurva	Himalaya: inner valleys to Arunachal Pradesh.
				J. communis	Kumaon westwards to Afghanistan.
				J. squamata	Afghanistan, Himalaya.
				J. indica	Northeast Burma.
				J. turkistanica	Himalaya: Indus to Sikkim, Bhutan Naga Parbat.
		Cupressus	2	*C. corneyana*	Tibet.
				C. cashmeriana	Unknown in wild, Ladakh (?).
Gnetaceae	1	Ephedra	7 + 3 var	*E. przewalskii*	Nanga Parbat, Ladakh
				E. pachyclada	Chitiral, Kurram valley.
				E. regeliana	Karakorum, Ladakh.
				E. gerardiana	Karakorum, Chitral, Gilgit, Baltistan, Kashmir, Ladakh, Zanskar.
				E. saxatilis var. sikkimensis	Tibet.
				E. nebrodensis var. procera	Kashmir.
				E. intermedia var. tibetica	Ladakh.

Results and Discussion

In present study through examination of herbarium specimens, literature investigation and extensive field surveys a total of 25 species of gymnosperms belonging to 13 genera, 6 families and 4 orders were documented from the Kashmir Himalaya (Table 16). Out of these, 11 species in 7 genera, falling within 4 families and 3 orders occur in wild (Table 14). The conifers with 9 species in 5 genera and 2 families form the most dominant group. Among families Pinaceae with 6 species in 4 genera is highest represented while Taxaceae with 1 species is least represented. It has been found that over the years several species of gymnosperms have been introduced and cultivated in gardens, parks, bare rocky

Table 12. Gymnosperm taxa reported from Kashmir Himalaya (Ara *et al*., 1995)

Name of family	Genus		Species		Distribution in Kashmir Himalaya
	Number	Name	Number	Name	
Ginkgoaceae	1	Ginkgo	1	G. biloba	Lalmandi.
Pinaceae	3	Pinus	4	*P. canariensis*	Shankaracharya.
				P. roxburghii	Uri, University Campus.
				P. halepensis	Shankaracharaya park, Bandipora, University
				P. radiata	Botanical Garden, Gulmarg. Shankaracharya.
		Cedrus	1	*C. deodara*	On lower altitudes in almost all forests, Chitarnar.
		Picea	2	*P. smithiana*	Afghanistan to Nepal
				P. spinulosa	-
		Abies	1	*A. pindrow*	All forest divisions, Yusmarg Dachigam, Tangmarg, Aharbal.
Taxodiaceae	2	Cryptomeria	1	*C. japonica*	University Botanical Garden, Shankaracharya.
		Sequoiadendron	1	*S. giganteum*	One old tree in Drug Research Farm Tangmarg.
Cupressaceae	2	Cupressus	3	*C. arizonica*	Parks and Gardens, University Campus. Parks and Gardens,
				C. sempervirens	University Campus. Generally growing between
				C. torulosa	2000-3000m, Chitarnar.
		Juniperus	3	*J. communis*	Zaberwan, Harwan,
				J. squamata	Parimahal, Gadsar, Wakulwan.
				J. wallichiana	Common above tree line, Gadsar. Gadsar, Apharwat.
		Thuja	1	*T. orientalis*	Gardens, Parks, Srinagar, Bandipora.
Taxaceae	1	Taxus	1	*T. wallichiana*	Zaberwan, Dachigam.

Table 13. List of Gymnosperm taxa reported from Kashmir Himalaya (Dar, 2004)

Name of order	Name of family	Name of genus	Name of species	Wild growing	Cultivated
		Pinus	*P. wallichiana*	+	-
			P. roxburghii	+	-
			P. halepensis	-	+
		Picea	*P. smithiana*	+	-
		Cedrus	*C. deodara*	+	-
		Abies	*A. spectabilis*	+	-
			A. pindrow	+	-
		Cryptomeria	*C. japonica*	-	+
		Sequoiadendron	*S. giganteum*	-	Single tree growing in Yarikhah Drug farm
		Cupressus	*C. torulosa*	-	+
			C. cashmeriana	-	+
			C. sempervirens	-	+
		Juniperus	*J. communis*	+	-
			J. squamata	+	-
			J. semiglobosa	+	-
		Thuja	*T. orientalis*	-	-
Taxales	Taxaceae	Taxus	*T. wallichiana*	+	Single shrub growing in Botanical garden
			T. baccata	-	Kashmir University
Ephedrales	Ephedraceae	Ephedra	*E. gerardiana*	+	-
Ginkgoales	Ginkgaoceae	Ginkgo	*G. biloba*	-	+
Total= 4	6	12	20	11	9

slopes and road sides in the State. Out of the total gymnosperms in our State, 14 species in 8 genera and 4 families and 2 orders occur in cultivation only (Table 15). Out of these, order Coniferales represents the dominant proportion with 13 species in 7 genera and 3 families while Ginkgoales is represented by only 1 species. Family Cupressaceae is most dominant with 9 species in 3 genera while as Ginkgoaceae is least represented (Table 15).

There is considerable difference of opinion as to whether there are one, two or more species of Himalayan silver firs. According to Troup (1921) the specific distinction in the Himalayan Silver fir is controversial in spite of the fact that two forms (*A. pindrow* Royle and *A. spectablis* Spach.) were raised true to seed. Turrill (1937) forwarded an explanation that it might be due to earlier evolutionary conditions from which *Abies alba* separated northwards and *Abies cephalonica* southwards through loss of different genes. Turrill's suggestion does not appear to be likely in the case of *Abies spectabilis* and *Abies pindrow*, as it involves the supposition that a new species is being evolved in more or less isolated locality. As per Brandis (1921) the low-level fir changes to high level species on ascending to higher ranges. Elwas and Henry (1906-1913) treated *A. spectabilis* Spach. as a mere variety of *A. pindrow* (*A. pindrow var brevifolia*).

Parker (1940) stated that the two species *viz. A. pindrow* and *A. spectablis* hybridize rather freely and that, in consequence, it is not always possible to make a sharp distinction between them. It seems likely that before the ice age there were two distinct Silver firs in the Northwest Himalaya and that during ice age they were forced to migrate. If change in climate was sufficiently rapid, one species might have invaded the zone of other and hybridization would have resulted in a mixed population with perhaps more or less complete submergence locally of one or the other species. As per author what is now occurring may be separation of the hybrid population into two species similar to original ones. The suspected hybrids can be recognized by upcurved tips of branches and trees with this character usually also show the hairy shoots of *A. spectabilis*, but they do not always do so. Parker further states that at some places there appears to be a polymorphic population showing the combination of these characters and this appears to be comparable with the intergrading of *A. alba* and *A. cephalonia* in Europe. This has been suggested to be due to hybridization. *Abies densa* Griff. which is treated as a separate species, is considered by

Table 14. Gymnosperms growing as wild in Kashmir Himalaya

Name of order	Name of family	Name of genus	No. of species	Name of species
Coniferales	Pinaceae	Pinus	2	*P. wallichiana*
				P. roxburghii
		Cedrus	1	*C. deodara*
		Picea	1	*P. smithiana*
		Abies	2	*A. pindrow*
				A. spectabilis
	Cupressaceae	Juniperus	3	*J. cummunis*
				J. squamata
				J. semiglobosa
Taxales	Taxaceae	Taxus	1	*T. wallichiana*
Ephedrales	Ephedraceae	Ephedra	1	*E. gerardiana*
Total = 3	4	7	11	

Table 15. Gymnosperms existing in cultivation in Kashmir Himalaya

Name of order	Name of family	Name of genus	No. of species	Name of species
Coniferales	Pinaceae	Pinus	1	*P. halepensis*
	Cupressaceae	Cupressus	6	*C. torulosa*
				C. cashmeriana
				C. guadalpensis
				C. gigantea
				C. sempervirens
				C. arizonica
		Juniperus	2	*J. chinensis*
				J. horizontalis
		Thuja	1	*T. occidentalis*
	Taxodiaceae	Cryptomeria	1	*C. japonica*
		Sequoia-dendron	1	*S. giganteum*
		Taxodium	1	*T. distichum*
Ginkgoales	Ginkgoaceae	Ginkgo	1	*G. biloba*
Total = 2	4	8	14	

Table 16. Summary of gymnosperm flora of Kashmir Himalaya

Name of family	Total number of genera	No. of genera in wild	No. of genera in cultivation	Total no. of species	No. of species existing in wild	No. of species existing in cultivation
Pinaceae	4	4	1	7	6	1
Cupressaceae	3	1	3	12	3	9
Taxodiaceae	3	0	3	3	0	3
Taxaceae	1	1	0	1	1	0
Ephedraceae	1	1	0	1	1	0
Ginkgoaceae	1	0	1	1	0	1
Total= 06	13	7	8	25	11	14

Dallimore and Jackson (1948) as a synonym of *Abies spectabilis* Spach. but Raizada and Sahni (1960) on the basis of their study of herbarium material are inclined to regard them as two distinct species.

Rao (1953) stated that hybridisation between *A. spectabilis* and *A. pindrow* in going on even today, because the zonal difference is narrow and Abies pollen is wind-dispersed. He argued that Turrill's idea corresponds to the concept of the cline (topocline, ecoline) but his postulate, 'loss of different genes' seems uncalled for. What he probably means is adapted mutations for a northern or a southern situation which after natural selection culminated in the divergence of two species. Yet these two species are compatible in hybridization, hence the intergrading forms. Parker wrongly interpret Turrill when he says, 'it involves the supposition that a new species is being evolved in a number of more or less isolated localities". Turill does not refer to present day divergence and species promotion but to an 'earlier evolutionary conditions'. If Parker is correct in doubting whether *A. spectabilis* and *A. pindrow* are two different species at all, then the altitudinal clinal concept will hold good, the two species being considered as only two clinal races which are quite compatible in hybridity. He further states that Parker's speculation that there were two distinct species before the ice age which hybridized under ice age conditions and that subsequent hybridizations is going on till today seems quite unnecessary. The hybridizations are going on today because the zonal difference is narrow and the *Abies* pollen is wind-dispersed. This question might be solved by raising self pollinated progeny of suspected hybrids and studying the segregation, if any.

Also, a lot of confusion exists regarding the *Juniperus* sp. occurring in the Kashmir Himalaya. Hooker (1888), Gamble (1902) and Parker (1924) recognized two species of *Juniperus* with scaly leaves occurring in the Himalaya *viz*., *J. pseudosabina* and *J. macropoda*. Hooker regarded *J. wallichiana* as synonymous of *J. pseudosabina*. However, Brandis (1874) recognized *J. pseudosabina* and *J. wallichiana* as separate species. These authors are of the view that *Juniperus wallichiana* is an evergreen tree attaining a height of 18m in Sikkim, but is a gregarious shrub in north-west Himalaya. Dallimore and Jackson (1966) hold the view that *J. pseudosabina* does not occur in India and the species that has been reported as occurring under this name is *J. wallichiana*. They have treated *Juniperus coxii* as a variety of *J. recurva*. However, none of the authors gave satisfactory criteria for delimitation of these two species.

Conclusion

In *Abies* complex, besides its two-parent species - *A. pindrow* and *A. spectabilis* there exist swarms of hybrids between these two species. These hybrids show various degrees of intermediacy in characters, such as bark, leaves (arrangement, apex notching, margin recurving, groove prominence), cone characteristic (axis length, thickness, swollen or pointed tips). In case of *Juniperus* complex, it has been found that *J. recurva* reported by previous workers (Sahni, 1990; Singh and Kachroo, 1976; Gaussen, 1968; Gamble, 1902; Hooker, 1888) from our area is actually *Juniperus squamata*. Furthermore, *J. macropoda* reported from northern Himalaya under various names (e.g *J. excelsa*, *J. polycarpos*) by various workers (Sahni, 1990; Raizada and Sahni, 1960; Lambert, 1933; Parker, 1918) has been found to be actually *J. semiglobosa*, an entirely distinct species. *J. pseudosabina* has been reported from Kashmir by many previous workers (Mehra, 1988; Gaussen, 1968; Lambert, 1933; Gamble, 1902; Hooker, 1888), but we have not found it in the valley. Also, *Juniperus chinensis* reported by Singh *et al*. (2018) to exist in wild in Kashmir Himalaya is doubtful, as it has been recorded to exist in cultivation only.

Acknowledgement

Authors are grateful to Head Department of Botany, University of Kashmir Srinagar and Principal Abdul Ahad Azad Memorial Govt. Degree College Bemina Srinagar for providing the necessary facilities to realize present sudy.

Conflict of Interest. The authors declare no conflict of interest.

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