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Review Article

Acute Health Consequences from Electronic Cigarettes: A Narrative Review

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Abstract: Electronic cigarettes (e-cigarettes) is relatively recently introduced to the market. Its long-term effects are still unclear and require years of research. On the other hand, knowledge about the short-term effects of e-cigarettes is emerging. The purpose of this review was to examine the current literature on the acute health consequences associated with the e-cigarette. We used pre-specified keywords to conduct searches in academic databases of articles published between 2011 and 2020. This review focused on acute health consequences of e-cigarettes in humans that can be directly attributed to the e-cigarette. Burns and injuries associated with device explosion, e-liquid intoxication, e-cigarette or vaping product use-associated lung injury, and pneumomediastinum were identified as acute health consequences associated with e-cigarettes. Except for pneumomediastinum, these acute health consequences are unique to an e-cigarette that are unlikely to be caused by a conventional cigarette. In the short term, the e-cigarette is likely more harmful than the conventional cigarette. Tightening safety regulations on the manufacturing, distribution, and sale of e-cigarette devices is recommended. Compulsory child-resistant containers for e-liquid containers, banning or restriction the use of colorful labels on e-liquid containers, and compulsory warning labels on e-liquid containers are recommended to prevent child's ingestion of e-liquid.

Keywords: Electronic Cigarette, E-cigarette, Health Consequence, Harm, Narrative Review

1. INTRODUCTION

Electronic cigarette (e-cigarette) is a battery-powered nicotine delivery device that does not generate side-stream smoke. Instead, it produces e-cigarette aerosol, often called vapor. In comparison to tobacco smoke, e-cigarette aerosol contains fewer chemical compounds at lower concentrations [1]. It has been marketed as a less harmful alternative to a conventional cigarette and has been used to facilitate smoke quitting [2].

The e-cigarette was introduced to the market around 2007 [2], which is slightly over a decade ago at the time of writing. As a result, its long-term effects on health have not been well-established. A study from Canada showed an increasing trend in past-month e-cigarette use among students Grade 9-12. Past-month e-cigarette use was associated with cigarette smoking initiation and subsequent daily cigarette use in this group [3]. A systematic review of nine prospective studies found an association between e-cigarette use and subsequent cigarette smoking initiation [4].

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According to the knowledge about the consequences of cigarette smoking, those who begin smoking before the age of 25 and continue to smoke will reach a point of disability due to deteriorated lung function, on average, at the age of 65 [5]. Smokers who began smoking during their adolescence will have an increase in the risk of chronic obstructive pulmonary disease (COPD) in their third decade of life. At the age of 60, the risk of developing COPD diagnosis is 7 % for males and 12 % for females. An increase in the risk of smoking-related heart disease is observed around the age of 40 [6]. A decade or two may be required to establish evidence of e-cigarette related chronic health consequences.

Hence, this study concentrates on the more established parts of the literature—i.e., acute health consequences associated with e-cigarettes. This evidence is emerging. Notably, the Centers for Disease Control and Prevention reported an outbreak of e-cigarette or vaping product use-associated lung injury (EVALI), an acute lung injury associated with e-cigarette use, in the United States in 2019 [7]. Several jurisdictions have recently tightened their regulations on e-cigarettes [8]. The purpose of this review was to examine the existing literature on the acute health consequences associated with the e-cigarette.

2. MATERIAL AND METHODS

2.1 Study design

The study design is a narrative review.

2.2 Data Source

We used pre-specified keywords to conduct searches in the Scopus, Web of Science, and MEDLINE databases. The keywords include "electronic cigarette", "e-cigarette", "electronic nicotine delivery system", "effect", "health effect", "harm", "acute health effect", and "acute harm". This review covered studies published between 2011 and 2020. In this review, e-cigarette does not include heat-not-burn tobacco. This review was conducted from 1st March to 31st July 2021.

2.3 Review Protocol

The studies included in this review must involve acute health consequences of e-cigarettes in humans. To be considered an acute health consequence, the duration between the exposure to e-cigarettes and the onset of such consequence must be 14 days or less, according to the Agency for Toxic Substances and Disease Registry's definition of "acute exposure" [9]. We included only health consequences that can be directly attributed to the e-cigarette. Both authors independently reviewed the titles and abstracts of articles from the database search to determine their eligibility. The original keywords search identified 1,015 studies; 942 studies were excluded by title and abstract screening. The full-text review included studies that were considered relevant by both authors. The consensus was used to resolve any disagreements regarding the inclusion of studies. During the full-text review, relevant studies listed in the bibliographies of included articles were added to the review. There were 158 full-text reviews and 63 studies were considered eligible by both authors.

3. RESULTS AND DISCUSSION

Four acute health consequences directly linked to e-cigarettes were identified. These include burns and injuries from device explosions, e-liquid intoxication, e-cigarette or vaping product use-associated lung injury (EVALI), and pneumomediastinum. Each component of an e-cigarette is associated with a specific consequence. The battery is a primary source of explosions. E-liquid contains a high concentration of nicotine, of which a few drops of ingestion can be fatal. Constituents of e-cigarette vapor contribute to EVALI development. Certain vaping-related behaviors cause an increase in intrathoracic pressure, which results in pneumomediastinum.

3.1 Burns and Injuries from Device Explosion

An e-cigarette device may spontaneously explode. The explosion potentially causes serious injury or death. We reviewed 39 incidents of burns and injuries caused by e-cigarette explosions from published case reports and case series (Table 1). The majority of cases were male (37 of these 39 cases). Their ages ranged from 15 to 59 years old. Most (84.6 %) were aged ≤ 40 years old.

The majority of explosions occurred when the e-cigarette was carried in the pocket of the pants

(21 of 39). This resulted in injuries to the lower extremities (particularly the thigh), genital area, and hand. In ten incidents, the explosions occurred while the victims were vaping. This resulted in the oral cavity (mucosa and teeth) and facial (maxillofacial fracture) injuries, as well as injuries to the eyes, vertebra, and brain. In three instances, the explosion occurred during or adjacent to the process of changing or charging the battery. Other incidents occurred when e-cigarettes were held in one's hand or placed in a bag/chest pocket. There was one fatality as a result of an e-cigarette explosion. A man's body was discovered in his house with 80 % of his total body surface area burned (TBSA). The x-ray of the head taken postmortem revealed that the e-cigarette device was lodged in the cranium and brain [10].

The majority of cases suffered from partial or full-thickness burns of affected body parts. The burning area varied from 1 to 80 % of TBSA. The thigh and other lower extremity areas were the most frequently affected body parts. Other affected body parts included the hand, buttock, genitalia, oral cavity, face, neck, eyes, and abdomen. Two cases of corneal burns occurred, impairing the affected individuals' vision [11]. Most cases were hospitalized for at least seven days.

Apart from these case reports, an attempt was made to estimate the number of emergency department visits caused by e-cigarette explosions in the United States using national data. Between 2015 and 2017, an estimated number of visits was 2035 in total [12]. The estimate for 2019 was 676 [13]. The authors noted that these figures were underestimated. Although considered rare in absolute terms, burns and injuries from e-cigarettes are significant enough that a group of plastic surgeons proposed a specific guideline for the management of e-cigarette burn injuries [14]. According to the guideline, inhalation injury and chemical burns must be considered in addition to burns and injuries from flame.

The battery in the e-cigarette device is thought to be the cause of the explosion. Lithium-ion batteries are used in the majority of e-cigarettes. The lithium-ion battery is vulnerable to a phenomenon known as "thermal runaway," which is a type of battery failure that results in overheating. This phenomenon can lead to an explosion of the battery [15, 16]. Mechanical, electrical, and thermal issues can lead to thermal runaway [16]. In the case of the e-cigarette explosion, it is unclear what triggers the phenomenon. As a result, the device's explosion is largely unpredictable. Cobalt, manganese, and other chemical substances are also found in some lithium-ion batteries. This may result in heavy metal toxicity and chemical burns, causing additional health problems for those who are affected [17]. This warrants more stringent e-cigarette device and battery regulations. The regulations may be complicated for jurisdictions that prohibit the use and sale of e-cigarettes, whereas a portion of the population still uses e-cigarettes, such as in Thailand [18]

3.2 E-liquid Intoxication

E-liquid is a liquid used with e-cigarettes that usually contains nicotine, propylene glycol, glycerin, and flavor [19]. The majority of e-liquids contain nicotine to simulate the sensation of smoking. Nicotine can be absorbed via alveoli, intestinal mucosa, oral mucosa, and nasal mucosa. Symptoms of acute nicotine toxicity include nausea, vomiting, diarrhea, sweating, tremor, confusion, irregular pulse, convulsion, respiratory failure, circulatory failure, and death [20]. Currently, there is no agreement on the lethal dose of nicotine. Previously, the oral LD50 of 0.8 mg/kg (equivalent to 60 mg of ingested nicotine) was thought to be lethal [21]. Recent studies have suggested significantly higher lethal doses, such as an oral LD50 of 6.5–13.0 mg/kg (corresponding to 500–1,000 mg of ingested nicotine) [21] and a plasma concentration of 0.8–1.6 mg/L (corresponding to 264–534 mg of ingested nicotine) [22]. Nicotine concentrations in e-liquid range from 10 to >200 mg/mL [23, 24]. As a result, ingesting just 1–5 mL of e-liquid with a high nicotine concentration could be fatal. Accordingly, e-liquid is a potentially hazardous product.

Nineteen cases of e-liquid intoxication were reviewed (Table 2). The age of these cases ranged from 15 months to 53 years. Ten were female. All incidents involving children aged 6 years or less were accidental, whereas most incidents in adolescents and adults involved suicidal attempts. Six cases had documented mental problems (depression, anxiety, and psychosis). Ingestion was the most frequently reported route of exposure, followed by intravenous injection in four cases.

In six instances, e-liquid was consumed alongside alcohol. Seven of these 19 cases resulted in death. Gastrointestinal symptoms like nausea, vomiting, and diarrhea were common in these cases. Ten cases involving severe conditions including respiratory failure, cardiac arrest, or death were found in ten cases. Two had seizures or seizure-like symptoms. Twelve cases provided enough data to estimate total nicotine intake, which ranged from 30 to 3,600 mg. Among the fatal cases in adults, nicotine intake ranged from 1,440 to 3,600 mg, which was higher than all of the estimated oral LD50 of nicotine. A 15-month-old patient died after consuming 500 mg of nicotine. One patient had a plasma propylene glycol concentration of 300 ug/L at 14 hours after ingestion of e-liquid. The initial propylene glycol level could be higher than 1000 mg/L, which could be toxic. E-liquid contains a variety of toxic substances in addition to nicotine. More toxicology research on the e-liquid solvent is required [25].

Using the United States national data, there were an estimated 1,555 emergency department visits of children who ingested e-liquid or used an e-cigarette in 2018–2019. Nearly 70 % of the cases involved children under two years old. Ingestion of food- or fruit-flavored e-liquid has been reported in some cases [13]. E-liquid poses a unique risk to children and adults. Regulations on nicotine concentration, flavor, solvents, and packaging are urgently needed.

3.3 E-Cigarette or Vaping Product Use-Associated Lung Injury (EVALI)

EVALI is a term that refers to an acute lung injury that occurs as a result of e-cigarette use. Its symptoms resemble those of pneumonia. The diagnosis of EVALI is established by ruling out other causes of pulmonary infection and lung injury plus the history of e-cigarette use within 90 days before the onset and presence of pulmonary infiltrate. Clinical manifestations of EVALI involve respiratory system (i.e., shortness of breath, cough, chest pain, tachypnea, and oxygen desaturation) and gastrointestinal system (i.e., nausea, vomiting, and abdominal pain). Additionally, fever, fatigue, and muscle pain are common manifestations [26].

Tetrahydrocannabinol (THC) and vitamin E acetate in e-liquid are associated with EVALI. Studies reported more than 80 % of patients with EVALI used THC with their e-cigarettes within 90 days of the onset [26, 27]. Vitamin E acetate was detected in bronchoalveolar-lavage fluid of more than 90 % of patients with EVALI [28]. A possible pathogenesis of EVALI is that volatile organic compounds, products of heating e-liquid, generate THC or vitamin E acetate carbonyl complexes, which are toxic to lung cells [29]. Exposure to e-cigarette vapor also leads to lung epithelial barrier dysfunction and damaged monocytes [30].

Since 2012, acute lung injury following e-cigarette vaping has been reported in the literature under a variety of different diagnoses [31, 32]. The diagnosis includes lipoid pneumonia, organizing pneumonia, and eosinophilic pneumonitis [31-33]. However, it received increased attention in 2019 following an outbreak of more than 200 cases of severe pulmonary disease linked to e-cigarette use in 25 states of the United States [7]. Thus, during this outbreak, the term “EVALI” was coined to refer to this condition [34]. Between August 2019 and January 2020, a total of 2,602 patients with EVALI were reported in the United States [27].

3.4 Spontaneous Pneumomediastinum

Pneumomediastinum, a condition characterized by the presence of free air in mediastinum, has been linked to e-cigarette use. There have been three cases of spontaneous pneumomediastinum developed after e-cigarette use reported in the literature (Table 3). All of these cases were male in their adolescent or early adult years. The symptoms include chest pain, dyspnea, dysphagia, neck stiffness, and voice change. The duration between vaping and symptom onset varied from immediately following vaping to one day. All three cases were hospitalized for two days and did not require surgical intervention. Two cases were followed up one week and two months after discharge. In both cases, no long-term effect was detected.

Pneumomediastinum occurs as a result of an increase in intrathoracic pressure, which results in air leakage from the alveoli [70]. Vaping-related behaviors such as deep inhalation [71], increased frequency of use [72], and a strong cough [73] may trigger this phenomenon.

Table 1. Case reports of burns and injuries from e-cigarette device explosion

Age (yr), sex	Location of e-cig. during explosion	Affected body part	Degree of injury	Reference
35, M	Changing battery	Hand	Deep partial-thickness and full-thickness burns	[35]
22, M	Charging battery	Hand and foot	1%TBSA, superficial partial-thickness	[36]
38, M	Found dead with an e-cigarette in the cranium	Head and body	Death caused by a projectile wound to the head from the explosion of a modified electronic cigarette device; 80 % of TBSA burns	[37]
20, M	Hold in hand	Face	1.5-cm depressed soft-tissue defect over the right nasal bone and 5-15 % of facial fractures	[38]
30, M	Hold in hand	Hand and fingers	2nd degree burns of the tip of the index finger, with mild first-degree burns to the middle and proximal phalanges and minor burns to the long and ring fingers	[39]
53, M	Hold in hand after changing the battery	Head, face, abdomen, and hand	First degree burns to abdomen, fracturing right thumb, maxillofacial trauma, and tooth fracture and avulsion	[40]
17, M	Stored in breast pocket	Right chest wall and palm	4 % TBSA, partial thickness burns	[17]
20, M	Stored in pant pocket	Left lower extremity	16 % TBSA, partial thickness burns	[17]
27, M	Stored in pant pocket	Right thigh and leg	10 % TBSA, partial and full thickness burns	[17]
29, M	Stored in pant pocket	Left lower extremity	5 % TBSA, partial and full thickness burns	[17]

Age (yr), sex	Location of e-cig. during explosion	Affected body part	Degree of injury	Reference
36, M	Stored in pant pocket	Left and right thigh	7 % TBSA, partial and full thickness burns	[17]
40, M	Stored in pant pocket	Left thigh	Severe burns at left posterior thigh	[41]
47, M	Stored in pant pocket	Left thigh, left hand, scrotum, and penis	9 % TBSA, partial thickness burns	[17]
19, M	Stored in pocket	Thigh and calf	7 %TBSA, partial and full thickness	[42]
Mid 20, M	Stored in pocket	Thigh and leg	6 % TBSA partial thickness burns	[43]
22, M	Stored in pocket	Thigh, scrotum, and hand	1%TBSA mixed depth burns to right thigh and scrotum; superficial partial thickness burns to left hand	[36]
23, M	Stored in pocket	Left thigh and left hand	5 % TBSA, partial thickness burns	[17]
29, M	Stored in pocket	Left and right thigh, scrotum, and penis	8 % TBSA, partial thickness burns	[17]
Early 30, M	Stored in pocket	Thigh, leg, and hands	10% TBSA, partial thickness burns; 2 -3 % TBSA, full thickness burns to left lower extremity	[43]
30, M	Stored in pocket	Thigh, and hand	3 %TBSA, partial thickness burns	[44]
30, M	Stored in pocket	Thigh and leg	8 %TBSA, partial thickness burns	[45]
31, M	Stored in pocket	Thigh, leg, and buttock	10 %TBSA, partial and full thickness burns	[46]
34, M	Stored in pocket	Leg	15 %TBSA, deep partial thickness and full thickness	[42]

Age (yr), sex	Location of e-cig. during explosion	Affected body part	Degree of injury	Reference
			burns	
35, M	Stored in pocket	Thigh	2 % TBSA, partial and full thickness burns	[42]
36, M	Stored in pocket	Thigh and hand	3 %TBSA, deep partial thickness and full thickness burns to right thigh and superficial partial thickness burns to right hand	[46]
39, M	Stored in pocket	Thigh and hand	4 %TBSA, partial thickness burns to right thigh and minor superficial burns to right hand	[44]
Early 40, M	Stored in pocket	Thigh, genitalia, and hands	3 -4 % TBSA, partial and full thickness burns to right thigh, scrotum, and penis; <1% TBSA partial thickness burns to bilateral hands	[43]
49, M	Stored in pocket	Thigh	7 %TBSA, superficial partial thickness burns	[36]
15, F	Stored in the bag	Hand and fingers	Partial and full thickness burns	[47]
16, M	Vaping	Eye, face, and neck	Facial and bilateral corneal burns, severe bilateral eye pain and vision impairment, facial and neck pain	[11]
18, M	Vaping	Oral cavity and abdomen	Oral and abdominal burns, oral lacerations, tooth fracture, and avulsion	[48]
18, M	Vaping	Oral cavity, face, hand, and tooth	Oral burns, laceration, and dental trauma	[49]
20, M	Vaping	Oral mucosa and teeth	Tooth pain and fracture and wound on oral mucosa	[50]
26, M	Vaping	Upper abdomen, shoulder, and chest	Burns and superficial skin pain to the shoulder, chest, and abdomen	[51]
28, M	Vaping	Oral cavity	Intraoral burns, tooth fracture, and avulsion	[52]

Age (yr), sex	Location of e-cig. during explosion	Affected body part	Degree of injury	Reference
30, F	Vaping	Face, oral cavity, hand, and neck	Fractured vertebrae, dental trauma, facial fractured, and hand with superficial lacerations	[53]
45, M	Vaping	Eye, face, and hand	Partial-thickness corneal laceration and bilateral corneal burns, first-degree facial and hand burns, vision impairment	[11]
59, M	Vaping	Head, face, and hand	Maxillofacial fractures, blurred vision, pneumocephalus, and dismissed hearing	[54]
27, M	Vaping after changing the battery	Neck, face, and teeth	Fractured vertebrae, partial-thickness burns to his lips and tongue, and fractures of bilateral upper incisors	[55]

Note: yr = year; e-cig. = e-cigarette; M = male; F= female; TBSA = total body surface area

Table 2. Case reports on e-liquid intoxication

Age, sex	Exposure	Co-intox.	Clinical findings	Serum nic. and cot. level	Outcome	Reference
15 mo, F	Accidental ingestion, 5 mL e-liquid (nic. conc. 10 mg/mL) Total nic. exposed: 500 mg	None	Vomiting, unconsciousness, anoxic brain injury	Nic.: Not reported Cot.: 1,716 ng/mL after 12 hr	Death	[24]
2 yr, F	Accidental ingestion, e-liquid (nic. conc. 24 mg/mL) Total nic. exposed: cannot estimate	None	Vomiting and irritability	Nic.: Not reported Cot.: Not reported	Survived	[56]
30 mo, F	Accidental ingestion Total nic. exposed: cannot estimate	None	Vomiting	Nic.: Not reported Cot.: Not reported	Survived	[57]
6 yr, F	Accidental ingestion, 10 mL e-liquid (nic. conc. 70.3 mg/mL)	None	Vomiting, diaphoresis, and copious secretions	Nic.: 348 ng/mL after 1 hr	Survived	[58]

Age, sex	Exposure	Co-intox.	Clinical findings	Serum nic. and cot. level	Outcome	Reference
	Total nic. exposed: 703 mg			Cot.: 742 ng/mL		
17 yr, F	Ingestion, 10 mL e-liquid (nic. conc. 210 mg/mL) Total nic. exposed: 2,100 mg	None	Tonic-clonic movement, and cardiac arrest	Nic.: Not reported Cot.: Not reported	Survived	[23]
19 yr, F (depression)	IV injection, undiluted e-liquid Total nic. exposed: cannot estimate	None	Cardiac arrest	Nic.: 0.002 µg/mL Cot.: Not reported	Death	[59]
21 yr, F	Ingestion, 30 mL e-liquid (nic. conc. 12 mg/mL) Total nic. exposed: 360 mg	None	Nausea, vomiting, pale skin, anxiety, hypotension, and miosis	Nic.: 95 µg/L Cot.: 2,800–4,400 µg/L	Survived	[60]
22 yr, F (mental health problem)	IV injection, 2 mL e-liquid (nic. conc. 18 mg/mL) with methadone Ingestion, 60 mL of the mixed solution Total nic. exposed: 30 mg (injection only)	None	Tachycardia, flushing, salivation, and nausea	Nic.: Not reported Cot.: Not reported	Survived	[59, 61]
23 yr, M	Ingestion with suicidal attempt Total nic. exposed: cannot estimate	None	Unconsciousness, bradycardia, and respiratory muscle paralysis	Nic.: 1,900 µg/L Cot.: 2,100 µg/L	Death	[62]
24 yr, F (depression)	Ingestion, 15 mL e-liquid (nic. conc. 100 mg/mL) Total nic. exposed: 1,500 mg	Alcohol	Cardiac arrest and brain damage	Nic.: >1,000 ng/mL Cot.: >1,000 ng/mL	Death	[63]
27 yr, M	Ingestion with suicidal attempt, e-liquid Total nic. exposed: 420 mg	Alcohol	Vomiting and sinus tachycardia	Nic.: 50 µg/L at 2 hr Cot.: 250 µg/L at 2 hr	Survived	[64]

Age, sex	Exposure	Co-intox.	Clinical findings	Serum nic. and cot. level	Outcome	Reference
27 yr, M	Ingestion with suicidal attempt, e-liquid (nic. conc. 16mg/mL and 18 mg/mL) equivalent to 23 mg/kg of nic. exposure Total nic. exposed: 1,380 mg	Alcohol	Seizure-like movement and cardiac arrest	Nic.: Not reported Cot.: Not reported	Survived	[23]
32 yr, F (depression and anxiety)	Ingestion, 10 mg e-liquid with attempted to suicide Total nic. exposed: cannot estimate	Alcohol	Dizziness, nausea, abdominal pain, and severe left ventricular systolic dysfunction	Nic.: Not reported Cot.: 7,069 ng/mL	Death	[65]
32 yr, M	Ingestion, 20 mL e-liquid (nic. conc. 72 mg/mL) Total nic. exposed: 1,440 mg	Alcohol	Brain hypoxia and cardiac arrest	Nic.: 1,600 ng/mL after 24 hr Cot.: Not reported	Death	[66]
32 yr, M (mental health problem)	IV injection with suicidal attempt, 4 mL e-liquid (nic. conc. 32 mg/mL) Total nic. exposed: 128 mg	Alcohol	Unconsciousness, respiratory failure, and bradypnea	Nic.: 500–800 µg/L after 2 hr Cot.: 1,300–2,400 µg/L after 2 hr	Survived	[60]
34 yr, M (psychosis)	Ingestion, 50 mL e-liquid (nic. conc. 72 mg/mL) Total nic. exposed: 3,600 mg	None	Death	Nic.: 5.5 mg/L in femoral, 136 mg/L in heart blood Cot.: 0.9 mg/L in femoral, 7.6 mg/L heart blood	Death	[67]
44 yr, M	Ingestion, 30 mL e-liquid Total nic. exposed: cannot estimate	None	Headache, nausea, abdominal pain, ventricular extra systoles, and tachypnea	Nic.: 21 µg/L after 13.5 hr Cot.: 102 µg/L	Survived	[25]

Age, sex	Exposure	Co-intox.	Clinical findings	Serum nic. and cot. level	Outcome	Reference
51 yr, M	IV injection with suicidal attempt, 10 mL e-liquid Total nic. exposed: 1,000 mg	None	Tachycardia, coma, bradypnea, mydriasis, myoclonus, flaccid paresia, and NSTEMI	Nic.: 12 µg/L Cot.: 3,210 µg/L	Survived	[68]
53 yr, M	Ingestion with suicidal attempt, 3 mL e-liquid Total nic. exposed: cannot estimate	None	Unconsciousness, diarrhea, vomiting, and cardiac arrest	Nic.: Not reported Cot.: 1,296 ng/mL	Survived	[69]

Note: co-intox. = co-intoxication; nic. = nicotine; cot. = cotinine; mo = month; yr = year; M = male; F= female; conc. = concentration; hr = hour; IV = intravenous; NSTEMI = non-ST segment elevation myocardial infarction

Table 3 Case reports of pneumomediastinum following e-cigarette use

Age (yr), Sex	Symptoms	Time from last vaping to onset	Treatment	Reference
17, M	Dyspnea and dysphagia	Immediately	Hospitalized for 2 days and discharged without specific treatment	[71]
20, M	Sudden chest pain after strong cough	2 hours	Hospitalized for 2 days and discharged without specific treatment	[73]
25, M	Substernal chest pain, neck stiffness, and voice change (to nasal voice)	1 day	Hospitalized for 2 days and discharged without specific treatment	[72]

Note. yr = year; M = male; F= female

4. DISCUSSION

Invented in 2003 and introduced to the market in 2007, the long-term health effects of an e-cigarette are not well-established [2]. Nonetheless, evidence regarding the acute consequences of e-cigarettes has emerged. This review identified four acute health consequences attributed to the e-cigarette. As with conventional cigarettes, a major cause of burn injuries, the e-cigarette can result in burns and injuries. Unlike conventional cigarettes, e-cigarette devices can explode on their own, whereas conventional cigarettes can explode when they come into contact with other explosive substances, such as ammonium nitrate [74]. Conventional cigarette contributes to the explosion frequently in higher-risk circumstances [74, 75], whereas the majority of e-cigarette device explosions occurred during normal daily activities (Table 1). Due to the unpredictable nature of e-cigarette device explosions, the implementation of prevention measures is challenging.

Nicotine intoxication from the conventional cigarette is extremely rare. Only two case reports of such incidents were found in the literature. In these cases, tobacco was extracted from nine and twenty conventional cigarettes and consumed in apparent suicidal attempts [76, 77]. One of these two cases was dead. In comparison, at least 19 cases of nicotine intoxication from e-liquid have been reported, resulting in seven deaths. Additionally, 1,555 cases of nicotine intoxication were estimated in the United States between 2018 and 2019 [13]. Ingestion of e-liquid can be both intentional and accidental. Accidental ingestion was common among children. As a result, e-cigarette contributes significantly more to nicotine intoxication than the conventional cigarette.

Short-term exposure to conventional cigarette smoking has a proinflammatory effect on lung tissues [78]. This enhances the risk of lung injury due to other causes, as demonstrated in animal models [78-80]. Conventional cigarette smoking in the short term is not associated with clinically significant lung injury [78, 79]. In comparison, short-term exposure to e-cigarette vapor can result in acute severe lung injury, referred to as EVALI. Since 2012, EVALI has been reported under different diagnoses [31, 32]. It is relatively common, as there has been a nationwide outbreak of EVALI in the United States since 2019 [7, 27]. In terms of acute lung injury, the e-cigarette is substantially more harmful than the conventional cigarette.

Three cases of spontaneous pneumomediastinum associated with e-cigarette use have been reported. This condition has been linked to illicit drug use, most notably marijuana and cracks cocaine [81-84]. A narrative review found that 29.6 % of pneumomediastinum cases had a smoking history [85]. Hence, an e-cigarette may be added to the list of risk factors for pneumomediastinum.

Device explosion, nicotine intoxication, and EVALI are all distinct acute health consequences associated with the e-cigarette. These conditions are unlikely to be caused by a conventional cigarette. Accordingly, the claim that e-cigarette is a safer alternative to conventional cigarette is losing ground. This review shows that e-cigarette is more harmful than a conventional cigarette in the short term. Further studies examining the risk-benefit of e-cigarette use in comparison to conventional cigarettes should be conducted using a short analysis time frame. According to this review, we recommend tightening safety regulations on the manufacturing, distribution, and sale of e-cigarette devices. To prevent child's ingestion of e-liquid, we recommend compulsory child-resistant containers for e-liquid containers, banning or restriction the use of colorful labels on e-liquid containers, and compulsory warning label on e-liquid containers.

5. CONCLUSION

This review identified four distinct acute harms associated with e-cigarettes: burns and injuries associated with device explosion, e-liquid intoxication, e-cigarette or vaping product use-associated lung injury (EVALI), and pneumomediastinum. Except for pneumomediastinum, three of these are unique health consequences of e-cigarettes. In the short term, the e-cigarette is likely more harmful than the conventional cigarette.

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7. CONFLICT OF INTEREST

The authors declare no competing interest.

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Research Article

Occurrence of *Artemisia chinensis* (L.) Plant (Asteraceae) in the Northeastern (Gilgit-Baltistan) Pakistan: Evidence from Molecular Phylogeny of nrDNA and cpDNA Sequences

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Abstract: *Artemisia chinensis* L. referred as *Crossostephium chinensis* (L.) Makino in the Flora of China is a rare and conceivably threatened plant species with an unclear origin in Asia. The species has been acknowledged so far from some islands of Taiwan. However, as it is extensively cultivated for ornamental and medicinal purposes in Japan, China, and the Philippines, it is still challenging to delimit its native range. This study confirms the presence of *A. chinensis* from Northern (Gilgit-Baltistan) Pakistan using molecular phylogenetic analysis and by assessing its distribution. The species were found in one site in the Skardu District of GB Pakistan and phylogenetic analysis indicated a close resemblance of the collected *A. chinensis* from the Skardu region with species of subgenus Pacifica of the genus *Artemisia* reported globally. According to the outcomes of the present study, it is proposed that broader field surveys should be conducted to acknowledge the distribution of *A. chinensis* plant from other districts of GB and cities of Pakistan as well. It is proposed that *A. chinensis* plant is present in North Pakistan and this plant should be mentioned and retained as rare species in the flora of Pakistan.

Keywords: Genus *Artemisia,* Asteraceae, *Artemisia chinensis* L., nrDNA and cpDNA Phylogeny, Gilgit-Baltistan, Pakistan.

1. INTRODUCTION

Artemisia L. is the largest plant genera of the Anthemideae tribe from the family Asteraceae with ~500 species frequently distributed in the Northern Hemisphere [1-4]. Based on floral and capitular characteristics, this genus was traditionally classified into 5 major subgenera-like subg. *Artemisia*, subg. Absinthium, subg. *Dracunculus* subg. *Seriphidium* and subg. Tridentatae [3]. Nevertheless, some inconsistencies were found in this traditional subgeneric classification due to incongruence with the latest molecular investigations on the genus *Artemisia* [5]. However, this classification is still extensively used and cited while dealing with the taxonomy and classification of this genus. Species of the *Artemisia* genus are very significant from both medicinal and economic points of view.

Plentiful secondary metabolites from extracts of *Artemisia* species have been reported for the treatment of certain health-related issues including anxiety, depression, epilepsy, insomnia, irritability, stress, and psychoneurosis [6]. *Artemisia* species hold crucial biological activities. The most prominent are antibacterial, antirheumatic, anthelmintic, antispasmodic, antimalarial, antitumor, antiseptic, hepato-protective [7-10], antidiabetic [11], antioxidant, and cytotoxic activities [12-14].

Additionally, an active drug Artemisinin [15-17] obtained from annual *Artemisia* species like *A. annua* is specifically used to cure malaria [18] and other deadly diseases [19-20]. Artemisinin discovery from *A. annua* was so for considered a noteworthy achievement in the field of ethnopharmacology and Physiology or Medicine category in 2015, this plant has been awarded a Nobel Prize [21]. Recently, the activity of *A. annua*

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against diseases COVID-19 and SARS-CoV-2 is under investigation [22]. *A. chinensis* is one of the rare species that belongs to the genus *Artemisia*.

This species was first described by Linnaeus [23] and is not in the *Crossostephium* Makino which is considered to be a monotypic genus and where this plant was frequently retained [24]. *A. chinensis* in Asian regions has been recognized as dispersed naturally in Taiwan, adjacent to southernmost Ryukyu, Orchid, Bonin, and the Islands of Okinawa. However, Ling et al. [25] questioned the native position of *A. chinensis* along with the Fujian, Zhejiang, and Guangdong coasts, but the suitability of climatic there was anticipated by the Maxent analysis. In China, Japan, and the Philippines, due to its extensive cultivation for ornamental and medicinal purposes, the origin or indigenous position of this plant is unclear and problematic. Hobbs and Baldwin, [24] believed that the distribution of *A. chinensis* plant is restricted to tropical regions because this plant lacks tolerance against cold.

The *Artemisia* genus was formally acknowledged with nearly 25 species [26] from Pakistan. An extensive taxonomic study by Hayat et al. [27, 28] on the genus *Artemisia* reported more species from the arid regions of Pakistan. However, the latest inquiries on *Artemisia* from Pakistan documented more species, especially from Northern Pakistan and the *Artemisia* genus now characterizes nearly 60 species from all subgenera from Pakistan except for the subgenus Tridentatae [29] *Tridentatae* species of the genus *Artemisia* are believed to be endemic only to North America and there is no evidence of the occurrence of subgenus *Tridentatae* species from other regions including Pakistan [27, 28]

This inquiry is the first attempt to report the presence of a medicinally important *Artemisia* species (*A. chinensis*) from the far-flung Skardu district of Gilgit-Baltistan Pakistan. This study also investigated the phylogenetic association of *A. chinensis* from Northeastern Pakistan with other *Artemisia* plants based on internal and external transcribed spacer (ITS and ETS) sequences of nrDNA and intergenic sequences (*psbA-trnH*) of cpDNA that sanctioned its taxonomic identity.

2. MATERIAL AND METHODS

2.1 Specimen Collection

Gilgit-Baltistan is situated in the Northeast of Pakistan between 34.6°–37.4°N, and 74°–77.5°E with an area of 45, 224 km^2. The maximum range of

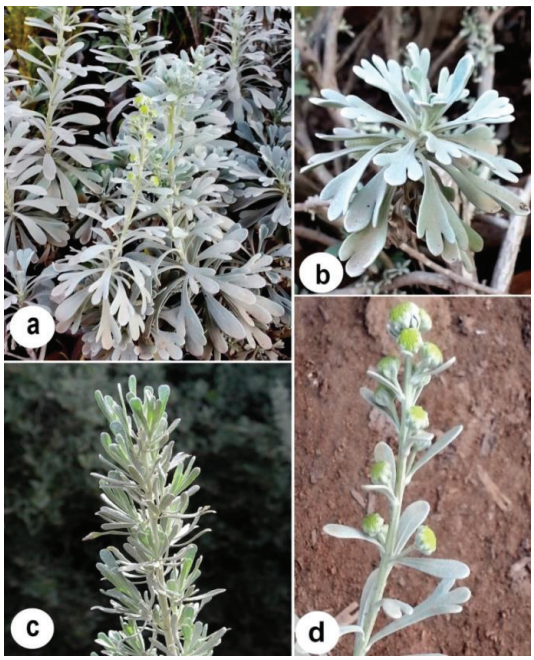

Fig. 1. Morphology of *A. chinensis* obtained from Skardu Gilgit-Baltistan Pakistan. a) Habit of the plant b) Basal leaves with lanceolate or spatulate 3 lobed blades, c) Aerial part with aggregated leaves, d) Inflorescence. Plant collection and original photographs by Adil Hussain and Tanseer Hussain

altitude of this region is 8611 m and the minimum is 1400 m. Gilgit-Baltistan has many districts and the major ones include Astore, Gilgit, Diamer, Ghizer, Ghanche, and Skardu Hunza-Nagar [30]. It has a temperate climate that is suitable for great plant diversity. In the course of the field samplings for the *Artemisia* project from the study area in 2016 and 2017 [31, 32], the occurrence of *A. chinensis* (Figure 1) was noticed in the Skardu District of Gilgit-Baltistan at a latitude of N-35′26.585 and longitude E-75′27.011. The sample was collected and the herbarium was consequently arranged (Figure 2). The specimen was deposited in the herbarium of Pakistan Museum of Natural History (PMNH) Islamabad Pakistan under the accession number PMNH-41722.

2.2 Morphology of Collected Plant

After assessing morphological characters and based on a BLAST search of molecular data, the species was recognized as *A. chinensis*. Various morphological characters of *A. chinensis* were evaluated in the dissecting and compound microscope with 4X, 10X, and 20X magnifications.

2.3 Phylogenetic Analysis

After collection, herbarium preparation, and morphological assessment of various features, the herbarium specimen of *A. chinensis* was transferred to the University of California Davis USA. From the exported herbarium specimens, leaf samples were taken to extract the total genomic DNA and to perform PCR for molecular phylogenetic analysis. The overall experimentation and data analysis were performed in the laboratory of Prof. Dr. Daniel Potter at the Department of Plant Sciences, University of California Davis CA, United States of America.

2.4 Extraction and Quantification of Genomic DNA

The leaf specimen was cleaned up with 70 % ethanol and the extraction of genomic DNA was done with a plant DNeasy kit (QIAGEN). After extraction, quantification of genomic DNA was done with the measurement of A260/280 values in a nanodrop spectrometer (ND-2000, Nanodrop Technologies USA) following Urreizti et al. [33]. 1.5% agarose gel was used and electrophoresis was performed to visualize the quality of extracted genomic DNA.

2.5 PCR Condition for the Amplification of ITS, ETS, and psbA-trnH regions

PCR was performed in the ABI thermo-cycle with 50 µl reaction volumes with ddH2O (36 µl), de¬oxyribonucleoside triphosphates (2 µl), 1xPCR buffer (5µl), MgCl2 (1µl), 1.5 µl both primers for ETS (18SETS and ETS-AST1), ITS (ITS9 and ITS6) and chloroplast *psbA-trnH* (trnHf and psbA3'f) (Table 1), template genomic DNA (1 to 1.5 µl of 20 to 50 ng), 0.5 µl of 5 units Taq polymerase and DMSO (1 µl). The optimized PCR amplification of ITS was achieved at 2 minutes pre-denaturation at 95 °C following 35 cycles with denaturation of 30 seconds at 95 °C, 1 minute annealing at 50 °C or 30 seconds annealing at 55 °C, and 72 °C, 1 minute extensions with the final extension of 5 minutes at 72 °C. The optimized PCR amplification of ETS was achieved at pre-denaturation for 2 minutes at 97°C with 36 cycles following denaturation for 2 sec at 97 °C, annealing at 55 °C for 30 seconds, and extensions at 72 °C for 30 seconds. A final extension was performed for 7 minutes at 72°C for ETS region amplification. The amplification of *psbA-trnH* sequence was performed at pre-denaturation of 5 minutes at 94 °C, following 30 cycles of denaturation for 1 minute at 94 °C, annealing of 1 minute at 55 °C, extension for 1.5 minutes at 72 °C. A final extension was achieved for 7 minutes at 72 °C.

The PCR products amplified were visualized and quantified in the electrophoresis containing agarose gel (1.5 %) arranged in 1xTBE with a voltage of 100 for 45 min in a buffer of Trisborate-ethylenediaminetetraacetic acid. The gel was then visualized under ultraviolet light in the trans-illuminator. During electrophoresis, PCR product size was perceived in comparison to the 1kb DNA ladder of standard size (Biolabs Company, N-3232L). The extraction of PCR product from the gel was carried out using a QIAGEN QIA-quick gel extraction kit with standard protocol.

2.6 Sequencing of PCR product and Multiple Sequence Alignment of Sequenced Data

The amplified DNA regions were then sequenced at the University of California Davis CA USA in a Big dye terminator version 3.1 cycle sequencing (ABI)

with capillary electrophoresis genetic analyzers (ABI 3730) using ETS, ITS, and *psbA-trnH* primers from both strands. The raw data of sequences of *A. chinensis* were assembled with software Sequencher. Total of four alignments (MSAs) were generated from ETS, ITS and *psbA-trnH* for new *A. chinensis* sequence and GenBank reference *Artemisia* species sequences were nrDNA-ITS (n=36), nrDNA-ETS (n=36), and cpDNA-psbA-trnH (n=36). One multiple sequence alignment (MSA) was obtained by combining ITS, ETS and *psbA-trnH* sequences (CAT-36; n=36). The details of multiple sequence alignments produced are given as;

MSA-1= ETS nrDNA (n=36) (1 new sequence+34 Gen¬Bank reference sequences+1 Outgroup sequence)
MSA-2= ITS nrDNA (n=36) (1 new sequences+34 Gen¬Bank reference sequences+1 Outgroup sequences)
MSA-3= *psbA-trnH* cpDNA (n = 36) (1 new sequences + 34 GenBank reference sequences+1 Outgroup sequences)
MSA4 = ETS nrDNA + ITS nrDNA + psbA-trnH cpDNA (CAT=36) (1 new sequence+34 GenBank sequences+1 Out¬group sequences)

Using the software MEGA-7 [34], these sequences were each aligned disjointedly followed by manual adjustments.

2.7 ML Phylogenetic Tree Construction

Primarily, 3 separate alignments were generated for ITS, ETS and psbA-trnH sequences (ETS with 390 characters, ITS with 653 characters, and psbA-trnH with 392 characters), and then the sequences of these three markers were combined [35, 36] to obtain final data matrix of 1435 characters. These separate and combined data matrices were analyzed using the maximum likelihood algorithm to find out the relationship of *A. chinensis* with other *Artemisia* species. The MEGA-7 software [34] was used to perform a maximum likelihood (ML) analysis and to visualize the final tree. *A. chinensis* sequenced

Fig. 2. *A. chinensis* herbarium specimen deposited at Pakistan Museum of Natural History (PMNH) Herbarium in Islamabad Pakistan (PMNH-41722

data with ITS, ETS, and *psbA-trnH* markers were submitted in the NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under accession number MH101881 for nrDNA ITS, MH292876 for nrDNA ETS and MH330169 for cpDNA *psbA-trnH*.

3. RESULTS AND DISCUSSION

The documentation of specimen PMHN-41722 (Figure 2) as *A. chinensis* characterizes the first record of this rare *Artemisia* species from Northern Pakistan. This species was recovered from one site in the district Skardu, Gilgit-Baltistan, Pakistan. The species was so for is recorded only near the Kachura/Shangrilla site in Skardu region of Gilgit-Baltistan Pakistan where it grows in grassy areas.

Morphological analysis showed the shruby nature of *A. chinensis* which is 10-40 cm tall (Figures 1 and 2). It has sometimes trailing branches with sessile leaves, aggregated at the top of the branches. It has densely gray-white pubescent. The blades of leaves are narrowly lanceolate or spatulate 2 to 4×0.4 to 0.5 cm thickly gray or white pubescent surfaces, cuneate-attenuate base, entire margin, apex 3 to 4 lobed and thick sometimes.

Small many disciform capitula with a diameter of 7 mm in a frondose raceme laterally branch. Hemispheric involucres and phyllaries are present in 3 rows where the outer and middle ones are elliptic, equal, and herbaceous. There is densely gray-white pubescent abaxially. The apex is acute or obtuse, the inner ones small, oblong, and subglabrous abaxially and the margin is scarious broadly. Female marginal florets are present in a single row which is tubular (ca. 1.5 mm), gland-dotted from outside with 2 or 3 denticulate apexes. Disk florets are many, tubular and 5-lobed, densely gland-dotted from the outer side. Fruits are conspicuously 5-ribbed and pappus is sometimes present with irregular teeth 0.5 mm, coroniform of small scales. The length of amplified DNA regions, raw generated sequences, multiple sequence alignments, and the numbers of informative sites for sequences of nuclear ribosomal DNA regions (ETS and ITS) and chloroplast DNA regions (*psbA-trnH*) for the species *Artemisia* are given in Table 2.

The data presented in the ML trees (Figures 3-6) based on ITS, ETS and *psbA-trnH* markers displays the dispersal of *A. chinensis* from Skardu district Gilgit-Baltistan Pakistan all through the clades corresponding to the other *Artemisia* species. All trees obtained from both independent and combined ML ETS, ITS, and *psbA-trnH* regions mended related topologies without any substantial conflicts. In the tree from independent data sets of each marker, *A. chinensis* from Skardu Gilgit-Baltistan was entirely supported which appeared in a single clade (ITS ML-BS = 97 %, ETS ML-BS= 64 %, *psbA-trnH* ML-BS= 82 %) covering species of the subgenus Pacifica including the previously reported *A. chinensis* from other parts of the world (ITS ML-BS = 100 %, ETS ML-BS= 86 %, *psbA-trnH* ML-BS= 99 %) as shown in Figures 3-5.

In a tree from combined data set of ITS, ETS, and *psbA-trnH* markers, *A. chinensis* from Skardu Gilgit-Baltistan was also completely supported in one clade (ML-BS = 100 %) with *A. chinensis* and other *Artemisia* species in a subgenus *Pacifica* clade reported from other parts of the world (ML-BS= 99 %) as shown in Figure 6. Consequently, the appearance of Pakistani *A. chinensis* in a clade (BS > 50 %) containing other *Artemisia* species, especially *A. chinensis* lineage from subgenus *Pacifica* of the genus *Artemisia* endorses its taxonomic identity. The plant species which are rare in nature have intrinsic, political, and ecological values that lead conservationists and land managers to ensure the protection of these plants. Keeping the rarity status of plant species, reserves are established in those areas where larger rare plant species are present [37-39]. It helps in the protection of the biodiversity contributions and the development of conservation strategies for rare plant species of the area [40].

A lot of studies reported the occurrence and native status of *A. chinensis* from different regions of the world [24, 46-48]. Studies concerning the presence of important phytochemicals having promising biological activities were also reported [49-52] *A. chinensis* was first described by Linnaeus [23] which is a monotypic genus, *Crossostephium* Makino in the flora of China and is a very popular traditional Chinese medicinal herb in Taiwan [48].

A. chinensis in Asian regions is thought to be distributed naturally in Taiwan adjacent to southernmost Ryukyu, Orchid, Bonin, and the Islands of Okinawa. However, Ling et al. [25] questioned the native position of *A. chinens*is along

Table 1. Details of three primers used for the amplification of nrDNA ITS, ETS, and cpDNA *psbA-trnH* sequences of the species of *Artemisia*

Markers	Marker name and Sequence	Length of base	References
ETS-forward primer	(AST1) 5′-CGTAAAGGTGCATGAGTGGTGT-3′	22	[41]
ETS-reverse primer	(18SETS) 5′ACTTACACATGCATGGCTTAATCT-3′	24	[42]
ITS-forward primer	(ITS9) 5′-GGAAGGAGAAGTCGTAACAAGG-3′	22	[43]
ITS-reverse primer	(ITS6) 5′-TCCTCCGCTTATTGATATGC-3′	20	
psbA-trnH-forward primer	(psbA3′f) 5′-GTTATGCATGAACGTAATGCTC-3′	22	[44]
psbA-trnH-reverse primer	(TrnHf-05) 5′CGCGCATGGTGGATTCACAATCC-3′	23	[45]

Table 2. Length of the PCR products and summary statistics of nrDNA ETS, ITS and cpDNA *psbA-trnH Artemisia* datasets. The numbers that appeared in the brackets specify the outcomes from the ingroup.

DNA Markers	ITS (nrDNA)	ETS (nrDNA)	*psbA-trnH* (cpDNA)	ITS+ETS +*psbA-trnH* (nrDNA + cpDNA)
Length of the PCR amplified region	~700 bp	~500 bp	~450 bp	
Samples number	36 (35)	36 (35)	36 (35)	36 (35)
Total number of sites	653	390	392	1435
Total number of informative sites	157(136)	96(83)	44(41)	300(257)

with the Fujian, Zhejiang, and Guangdong coasts, but the suitability of climatic there was anticipated by the Maxent analysis.

The monotypic *C. chinensis* was considered as a distant genus from the genus *Artemisia* [53]. It is believed that this genus is different from *Artemisia* due to the coroniform pappuspresence, however, it was formerly positioned with *Artemisia californica* by Rydberg [54] and Gray [46] on a deeply ribbed cypselas basis.

Gray [46] formerly put forwarded a close relationship of Hawaiian *A. australis* and *A. chinensis* earlier to the account of additional Hawaiian species. Conferring to Watson et al. [47] ITS phylogenetic analysis, the *C. chinensis* (*A. chinensis*) is allied with Old World Seriphidium and numerous species of subgenus *Artemisia* advocates that *C. chinensis* (*A. chinensis*) is unified within *Artemisia*.

Molecular phylogenetic study of Hobbs and Baldwin, [24] displayed a robust relationships resolution of the South Asian *A. chinensis* and Hawaiian *Artemisia*. They proposed a clade containing Hawaiian *Artemisia* and *A. chinensis* a new subgenus called *Artemisia* subgenus *Pacifica*.

This new subgenus Pacifica contains *A. chinensis* from littoral habitats in Southeast Asia, for example, Okinawa, Taiwan, Bonin, and Ryukyu islands, and 3 species viz *A. kauaiensis, A. australis,* and *A. mauiensis* of subalpine to littoral habitats in Hawaiian Islands [24]. Species of this subgenus *Pacifia* appeared monophyletic in the phylogenetic investigation of Malik et al. [55] concerning the taxonomy and classification of the subg. Seriphidium of the genus *Artemisia*

Hobbs and Baldwin [24] displayed that the nuclear ribosomal DNA and chloroplast DNA sequences support the hypothesis that the Southeast Asian *A. chinensis* is closely allied to the Hawaiian *Artemisia* taxa, which also make a clade. Results of this study also showed a close relationship between *A. australis* and *A. chinensis* under the subgenus Pacifica clade from the Northeastern Skardu region of Pakistan.

Presently, *A. chinensis* has been declared as possibly threatened in the Flora of China. Based on the rarity and less population, *A. chinensis* reported here from Northeastern Pakistan should also be declared a rare and possibly threatened species in the flora of Pakistan.

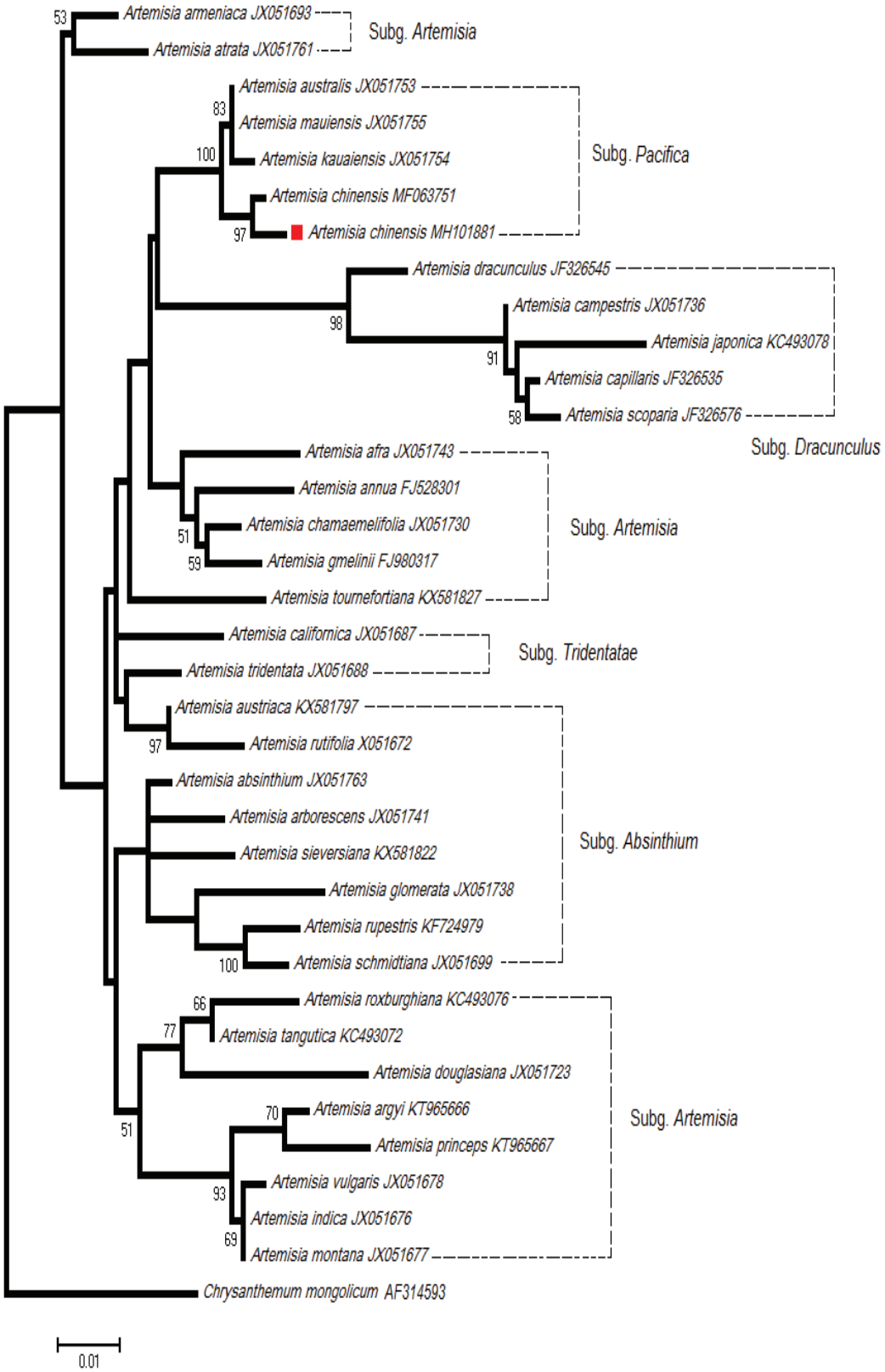

Fig. 3. Maximum likelihood phylogenetic tree constructed based on nrDNA ITS sequences of *Artemisia.* Values displayed above branches are the bootstrap values acquired from ML analysis with 1000 replicates. Colored shape denotes *A. chinensis* sequence from the Skardu region of Gilgit-Baltistan Pakistan. The subgeneric classification of the genus *Artemisia* following Bremer [56], Torrell *et al*. [1], Valles *et al*.[57], Sanz *et al*. [2], Garcia *et al.* [4], Pellicer *et al.* [58], Riggins and Seigler [59], Hobbs and Baldwin [24], Malik *et al*. [55] is indicated with vertical bars.

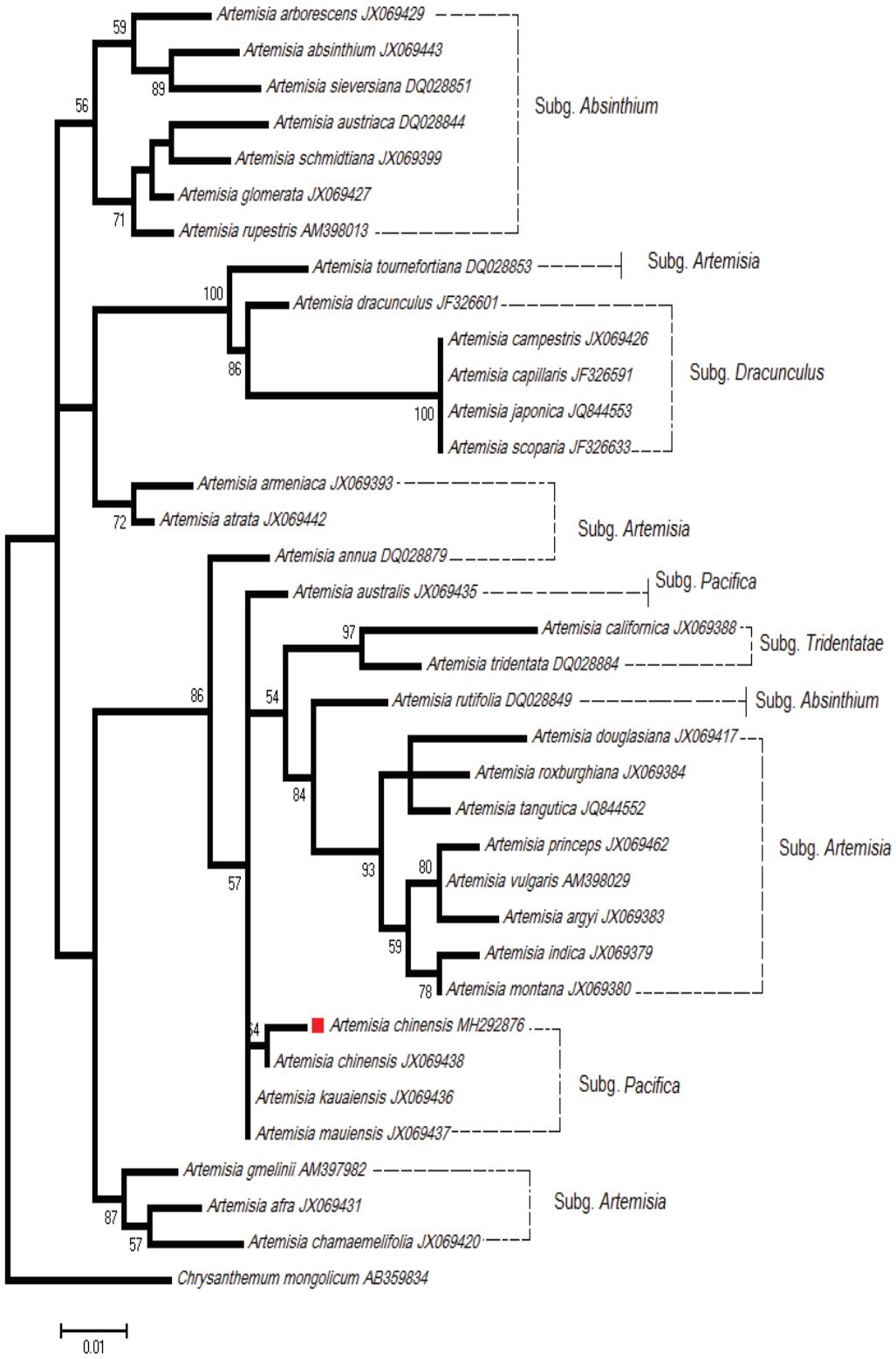

Fig. 4. Maximum likelihood phylogenetic tree constructed based on nrDNA ETS sequences of *Artemisia*. Values displayed above branches are the bootstrap values acquired from ML analysis with 1000 replicates. Colored shape denotes *A. chinensis* sequence from the Skardu region of Gilgit-Baltistan Pakistan. The subgeneric classification of the genus *Artemisia* following Bremer [56], Torrell *et al.* [1], Valles *et al.*[57], Sanz *et al.* [2], Garcia *et al.* [4], Pellicer *et al.* [58], Riggins and Seigler [59], Hobbs and Baldwin [24], Malik *et al.* [55] is indicated with vertical bars

Fig. 5. Maximum likelihood phylogenetic tree constructed based on cpDNA *psbA-trnH* sequences of *Artemisia*. Values displayed above branches are the bootstrap values acquired from ML analysis with 1000 replicates. Colored shape denotes *A. chinensis* sequence from the Skardu region of Gilgit-Baltistan Pakistan. The subgeneric classification of the genus *Artemisia* following Bremer [56], Torrell *et al*. [1], Valles *et al*. [57], Sanz *et al*. [2], Garcia *et al*. [4], Pellicer *et al*. [58], Riggins and Seigler [59], Hobbs and Baldwin [24], Malik *et al*. [55] is indicated with vertical bars.

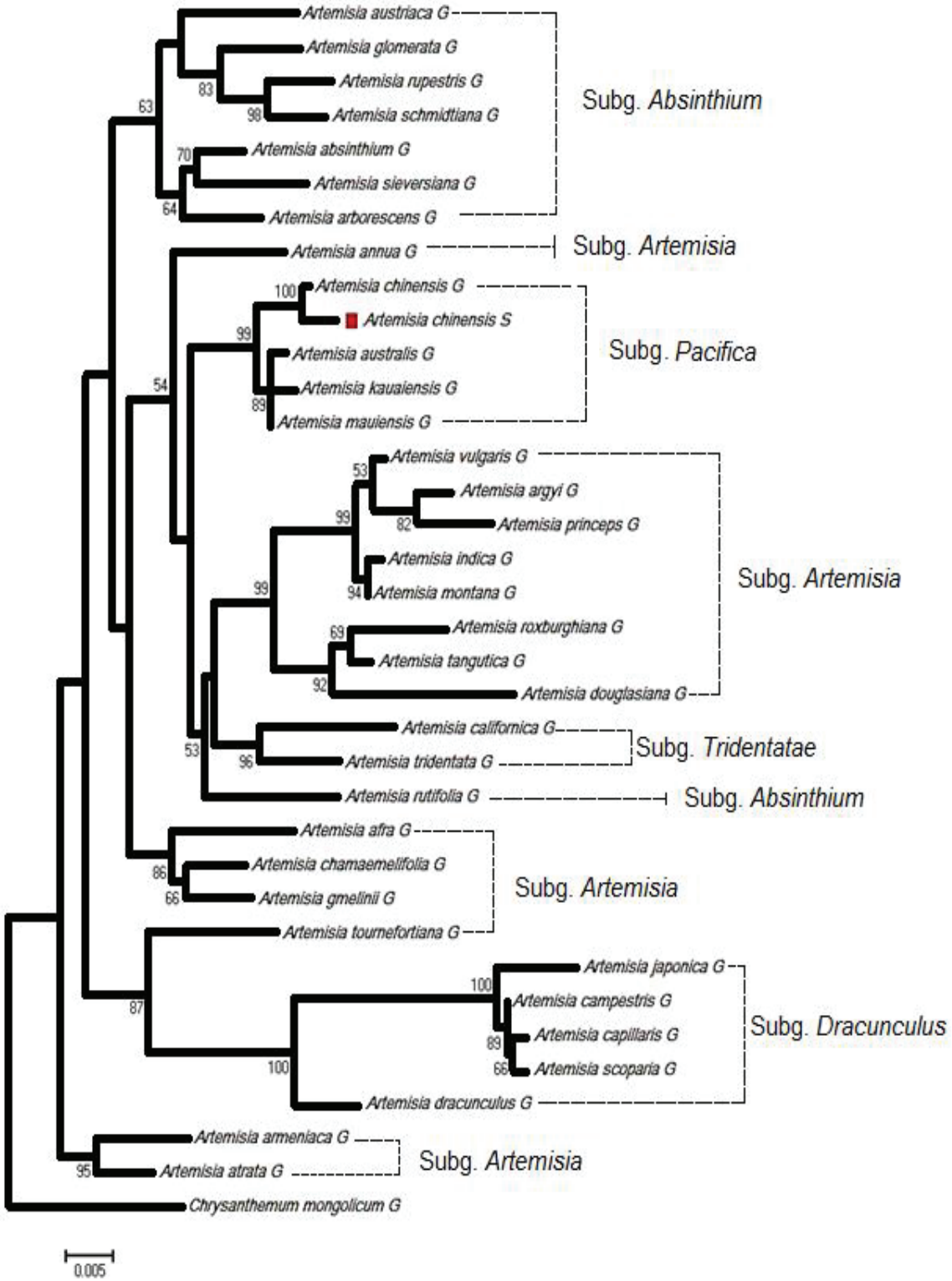

Fig. 6. Maximum likelihood phylogenetic tree constructed based on nrDNA+cpDNA (ITS+ETS+*psbA-trnH*) sequences of *Artemisia.* Values displayed above branches are the bootstrap values acquired from ML analysis with 1000 replicates. Colored shape denotes *A. chinensis* sequence from the Skardu region of Gilgit-Baltistan Pakistan. The subgeneric classification of the genus *Artemisia* following Bremer [56], Torrell *et al*. [1], Valles *et al*. [57], Sanz *et al*. [2], Garcia *et al*. [4], Pellicer *et al*. [58], Riggins and Seigler [59], Hobbs and Baldwin [24], Malik et al. [55] is indicated with vertical bars.

4. CONCLUSION

The occurrence of *A. chinensis* in the Skardu GB region of Pakistan stated in the present study permits the necessity of wide-ranging sampling to confirm its distribution not only from other sites of the Gilgit-Baltistan region but across Pakistan. It is assumed that *A. chinensis* plant may be also present in other parts of the GB, but not been reported by researchers and its presence could also be evident in other localities of Pakistan as well. Based on the outcomes of this study, it is proposed that *A. chinensis* plant is present in North Pakistan and this plant should be retained as rare species in the flora of Pakistan.

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6. CONFLICT OF INTEREST

The author declare no conflict of interest.

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Research Article

Parasitism performance of *Aenasius bambawalei* (Hayat) on *Phenacoccus solenopsis* (Tinsley)

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Abstract: The *Aenasius bambawalei* Hayat (Hymenoptera: Encyrtidae) is a solitary nymphal endoparasitoid of mealybug, *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae). The parasitism performance of *A. bambawalei* on the 3rd nymphal instar of *P. solenopsis* at different densities 20, 30, 40 and 50 *P. solenopsis* was tested. The results showed that the maximum number of *P. solenopsis* parasitized and male and female adults emerged at 30 *P. solenopsis* density than other densities. Similarly, the maximum sex ratio M: F (1: 1.7) was found at 50 *P. solenopsis* density as compared to others. The highest parasitization percentage was observed on 3-day old *A. bambawalei* at all *P. solenopsis* densities. The 30 *P. solenopsis* density is the most preferred and suitable for mass rearing of *A. bambawalei* and the 3-day old parasitoid use of biological control programme to control *P. solenopsis*.

Keywords: *Aenasius bambawalei*, *Phenacoccus solenopsis*, Parasitism, Densities

1. INTRODUCTION

Cotton mealybug (*Phenacoccus solenopsis*), originated in Central America, is considered a widespread pest in Pakistan. It has been reported from China and India destroyed the cotton industry in the previous few years [1-2]. In Pakistan, *P. solenopsis* appeared in 2005 first time, reported in Districts Sanghar and Vehari, Pakistan, during 2006 and 2007 the economic losses of cotton were 40 % and reached 3.1 million bales affected by this pest, which was the most destructive. *P. solenopsis* damaging has distributed throughout the growing fields of cotton in Pakistan and it has become a major insect pest of cotton and several important crops [3].

It is a highly polyphagous mealybug species attacking more than 200 plants including field and medicinal plants, ornamentals, weeds, and horticultural crops [4-6]. It is infested the fruits, leaves, main steams, branches, roots, and trunks feeding on phloem sap [5]. Mostly leaves and shoots are damaged by the huge amount of honeydew production that is responsible for the growth of sooty mold. [7].

P. solenopsis is difficult to control with pesticides since it is protected by a hydrophobic wax layer that acts as a barrier to penetration of pesticides [7]. Applied and natural biological control methods are essential in the successful management of insect pests. It is well known that natural enemies of insect

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pests play a vital role in biotic balance, reducing the level of insect pest population below the economic injury level. Mostly synthetic pesticides reduce the beneficial predators and parasitoids. The value of biocontrol is now well recognized, principally in the context of environmental protection as well as the sustainable strategy of pest management [8].

Several parasitoid species play a vital role in biological control methods and the Encyrtids are known as effective parasitoids of mealybug [9-10]. *Aenasius bambawalei* is a primary parasitoid of *P. solenopsis* in various regions of the world like India [11-13], southern China [14], Pakistan [15], and Iran [16]. It is an effective nymphal solitary endoparasitoid of *P. solenopsis* and due to the natural conditions, there is a good mortality factor for controlling this pest. It has been recognized that parasitism has a profound effect on population growth, development, and host fecundity. In Southwestern Iran, the parasitism percentage of *P. solenopsis* through *A. bambawalei* was up to 95 % in 2012 when insecticides were not used to control this pest [16].

The present study was designed to determine the parasitism performance of *A. bambawalei* on the 3rd nymphal instar of *P. solenopsis* (a) effect of different *P. solenopsis* densities of on *A. bambawalei* parasitization, adult emergence, and their sex ratio (b) parasitization effect of different *A. bambawalei* ages on different densities of *P. solenopsis*.

2. MATERIAL AND METHODS

2.1 Insect Collection and Rearing

The experiment was conducted at the Biocontrol laboratory of Entomology Section, ARI, Tando Jam, during 2020-21. The mummies or parasitized mealybug was collected directly in plastic jars from the field of (Tomato, okra, cotton, and Abutilon theophrasti weed plant) surrounding Sindh Agriculture University, Tando Jam, and shifted in plastic glass jars. After the full emergence of *A. bambawalei* healthy parasitoids were released on its host colonies of *P. solenopsis*. The culture of the parasitoid and its host were continued in plastic glass jars, at 27 ± 2 °C with 60 – 70 % Relative Humidity. After freshly emerged adult parasitoid female and male were taken from the reared culture and allowed for 24 hrs to mate and 50 % honey and 50 % water of solution was also provided as a source of food.

2.2 Experimental Design

The laid out experiment was a Completely Randomized Design with four treatments and each treatment with three replications. The freshly emerged female and male adult parasitoid were paired together for allowed to mate (24 hrs). After the next day's mating, the healthy mated female of the parasitoid was transferred or released in glass jars along with mealybugs on the leaf of abutilon weed for parasitization. After two days females were removed from the plastic jars. The parasitized host mealybugs were reared on tomato and abutilon leaves. Used camel brush and daily clean the jars to avoid contamination of diseases. The data of parasitization were recorded daily of adult parasitoid life and adult emergence and sex ratio of male or female also recorded. The 2nd experiment (1 day, 3 days, 7 days, and 15-day old parasitoid) at 24 hrs mated female was provided to 3rd nymphal instar of the host was four different densities 20, 30, 40, and 50 mealybugs in each glass jars. The data of parasitization were recorded after 24 hrs. The parasitization percentage was calculated through the formula is;

$$\text{Parasitization \%} = \frac{\text{No. of } P.\,solenopsis \text{ parasitized}}{\text{Total } P.\,solenopsis} \times 100\ \%$$

2.3 Statistical Analysis

The data was subjected through ANOVA (analysis of variance) using 8.1 Statistix software and the significant difference between the means of treatments was compared by LSD at $P \leq 0.05$.

3. RESULTS

The results indicated the number of mealybugs parasitized, parasitization percentage of male and female adult emergence, and sex ratio of *A. bambawalei* shown in Table 1. The highest number of *P. solenopsis* parasitized was recorded (305 ± 17.6) at 30 *P. solenopsis* density and the lowest number of *P. solenopsis* parasitized

(211 ± 13.3) was observed at 20 *P. solenopsis*. Similarly, the maximum parasitization of 42.2 % was recorded on 20 *P. solenopsis* density and the minimum parasitization of 18.9 % was observed on 50 *P. solenopsis*. However, the highest male and female adult emergence (120 ± 7.9 and 167 ± 15.7) was observed on 30 *P. solenopsis* density and the lowest male and female adult emergence (74 ± 4.9 and 116 ± 7.8) was recorded on 20 *P. solenopsis* density. Similarly, the maximum sex ratio M: F (1:1.7) was recorded at 50 P. solenopsis density and the minimum sex ratio M: F (1:1.4) at 30 *P. solenopsis* density.

The results regarding parasitization efficiency of different ages of *A. bambawalei* (1 day, 3 days, 7 days, and 15-day old parasitoid) on different densities of *P. solenopsis* (Fig. 1). However, the highest parasitization percentage was observed on 3- day old *A. bambawalei* at all *P. solenopsis* densities and lowest parasitization percentage was observed on 15-day old *A. bambawalei* at all *P. solenopsis* densities. The best results were found on 3-day old parasitoid at 30 *P. solenopsis* density with 71.1 %.

4. DISCUSSION

The present study aimed to examine the Parasitism performance of *A. bambawalei* on the 3rd nymphal instar of *P. solenopsis* at different densities 20, 30, 40, and 50 mealybugs at under laboratory

Table 1. Effect of different *P. solenopsis* densities on *A. bambawalei* parasitization, adult emergence and sex ratio.

Densities of *P. solenopsis*	No of P. solenopsis Parasitized	Parasitization (%)	Adult emergence		Sex ratio
			Male	Female	M: F
20 *P. solenopsis*	211 ± 13.3[b]	42.2	74 ± 4.9[b]	116 ± 7.8[b]	01:01.6
30 *P. solenopsis*	305± 17.6[a]	40.7	120 ± 7.9[a]	167 ± 15.7[a]	01:01.4
40 *P. solenopsis*	296±16.7[a]	29.6	110 ± 6.5[a]	162 ± 8.0[a]	01:01.5
50 *P. solenopsis*	236±15.6[b]	18.9	81 ± 6.9[b]	134 ± 6.3[ab]	01:01.7

Means followed by the same letters in similar columns are not significantly different (P > 0.05).

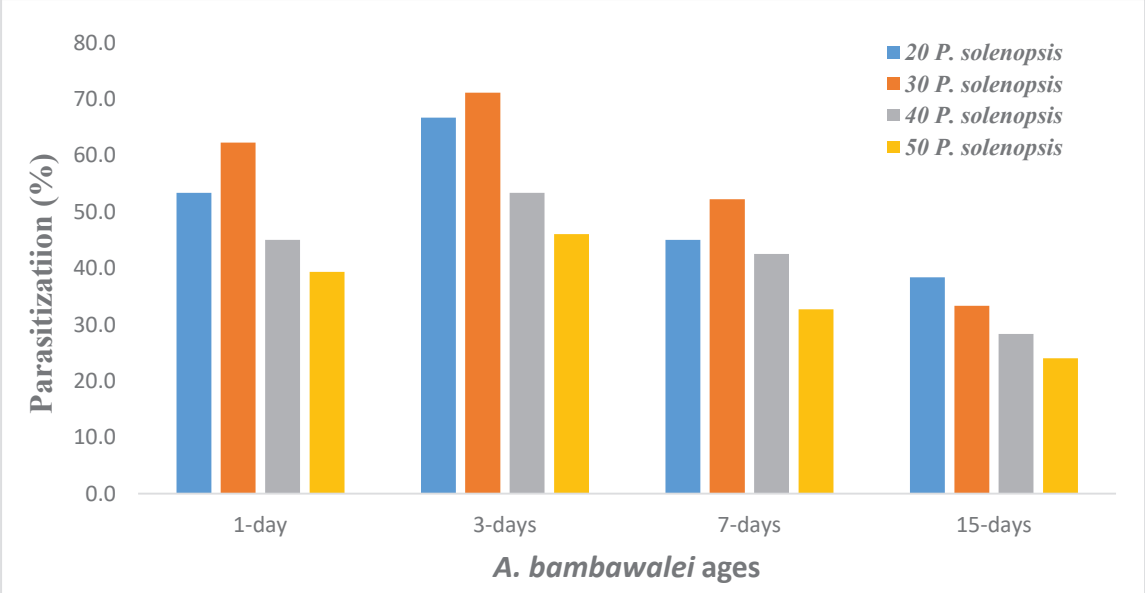

Fig. 1. Parasitization (%) of *A. bambawalei* different ages on *P. solenopsis* different densities.

conditions. All the parameters such as the number of *P. solenopsis* parasitized, parasitization percentage, adult emergence, and sex ratio of *A. bambawalei*. [17] studies on natural enemies of the mealybug (*P. solenopsis*) have reported a huge number of *A. bambawalei* on the different instars of mealybug. They also reported that the third nymphal instar of *P. solenopsis* is the most suitable stage of host for *A. bambawalei* mass rearing.

The results are supported by many past researchers, Zhang *et al*. [18] also reported that the maximum parasitization was on the 3rd nymphal instar of *P. solenopsis*. The highest number of the parasitized mealybug was recorded at 30 density and the lowest number of *P. solenopsis* parasitized was observed at 20 density. These results are in accordance with previous findings of Joodaki *et al*. [19], they reported that the maximum number of parasitized *P. solenopsis* nymphs by *A. bambawali* was 9.4 at density (64 nymphs) and reached a minimum of 1.8 at a density (2 nymphs). However, the parasitized nymphs increased with increase the number of hosts. Feng *et al*. [14] reported that the parasitized *P. solenopsis* increased with increased *P. solenopsis* density.

In the present study, the maximum parasitization percent was found on 20 *P. solenopsis* density and the minimum parasitization percent was observed on 50 *P. solenopsis*. Similar results were observed by Kumar *et al*. [11] and Ram and Saini [20]. The highest number of adults were found at 30 densities of *P. solenopsis* and the lowest male and female adults emerged at 20 *P. solenopsis* density. Shahzad *et al.* [21] and Iftikhar *et al*. [22] reported similar results the highest emergence of the parasitoid in the 3rd nymphal instar of mealybug.

However, the maximum sex ratio M: F (1: 1.7) was recorded at 50 *P. solenopsis* density, and the minimum sex ratio M: F (1: 1.4) 30 *P. solenopsis* density. Similar findings, the highest sex ratio of female wasps was recorded at the third instar mealybug nymph and the maximum male sex ratio was observed at the second instar host stage [23-25]. However, the highest parasitization percentage was observed on 3-days old *A. bambawalei* 71.1 % at 30 *P. solenopsis* density and lowest parasitization percentage were observed on 15-days old *A. bambawalei* 24.0 % at 50 *P. solenopsis* density. Similarly, Ignacimuthu and Jayaraj [8] also observed that 5-days after the emergence of *A. vexans* parasitoid highest number of mealybugs parasitized.

5. CONCLUSIONS AND RECOMMENDATIONS

The present study confirmed that *A. bambawalei* parasitized the *P. solenopsis*. They significantly decreased the third instar of mealybug. Thus, it was confirmed through this study *A. bambawalei* will be useful to control *P. solenopsis*. Further study is much needed to observe the complete functional response of the parasitoid on various densities of different insect pests.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Short-Term Impact of Plant and Liquid derived Fulvic Acids on the Physiological Characteristics, Plant Growth and Nutrient Uptake of Maize-Wheat Production

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Abstract: Fulvic acids (FAs) are the major component of soil organic matter, which improves soil structure and fertility. FA has been observed positively on plant growth and ultimately enhances crop production. The pot experiment was conducted on wheat and maize grown in silty clay, sandy loam, and clay loam textural soils, respectively. Three different parent materials; FA Solid (S=Powder Form), Natural Liquid (NL) and Plant-derived Liquid (P) were applied at 0% (Control), 0.25 % (S), 0.50 % (NL) and 0.50 % (P) FA, respectively. The results showed that the stem diameter of maize was 15.68, 26.90, and 26.35 mm under S, P, and NL respectively, however, the spike weight of wheat was 123.24, 98.5, and 132.4 g pot-1 for S, P, and NL in Albic (AL), Irrigated Desert (IR) and Shahjiang (SH) soils. Similarly, maize height increased by 8 % and 9 % significantly as compared to control and the height of wheat increased by 4 % and 1 % in AL and 5 % in IR soil compared to control. Maize grain weight increased over control; however, wheat grain weight significantly decreased. The N and P significantly enhanced in maize and wheat in AL, IR, and SH soils. Our study proved that the application of Solid FA did not improve maize growth characteristics, however, it improved the characteristics of wheat crops except under IR soils. In contrast, liquid FA improved the chemical and physical properties of soils including nutrient uptake of maize and wheat under AL and SH soils.

Keywords: Fulvic acids; Mazie; plant growth characteristics; Nutrient uptake; Wheat

1. INTRODUCTION

Fulvic acid (FA), the major component of soil organic matter, is the subject of study in various areas of agriculture, such as soil chemistry, fertility, plant physiology as well as environmental sciences, because of the multiple roles of this material which can greatly benefit plant growth [1, 2]. The positive properties of FA on plant growth may be attributed to its increase in fertilizer efficiency or enhancement

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of plant biomass [3]. In specific, the root growth increase is generally more obvious than that of root growth [4]. FA has the potential to mitigate the stress of the heavy metal on plant growth [5] and enhance the yield by increasing the nutritional status of soil [6]. FA correlated at efficient concentration sustained the Fe and Zn in solution. In this context, FA has been widely considered as performing a valuable role in Fe acquisition by plants [1,7]. The FA effect is ascribed to the complexing properties of FA, though micronutrients availability significantly increases from soluble hydroxides [8]. Plant metabolism is positively modified with a low mass of FA [4]. Their effects appear to be mainly exerted on cell membrane functions, promoting nutrient uptake [7, 9] and/or plant growth and development, by acting as hormone-like substances [4,10]. While it was also studied that FA increases the chlorophyll content, accelerates plant respiration and hormonal growth responses, increases penetration in plant membrane, etc. Similarly, FAs are also vital for the nutrient uptake of plants. Firstly, they serve as a source of N, P, and K [8] and S [11] through mineralization by soil micro-organisms, and secondly act as the organic matter which influences the supply of nutrients and improves soil properties. Carboxyl and hydroxyl groups of FA significantly increase crop production and plant metabolism and improve respiration activities [12]. Tahir *et al.* observed similar results [13] and they found that plant growth and shoot weight of wheat plants increased when the FA dose was 60 mg kg^{-1}. The same tendencies were reported by Sharif *et al.* in a pot experiment of Maize, he concluded that humic acid significantly increases the shoot weight when applied at 50 mg kg^{-1} [14]. Similarly, Çelik *et al.* reported that when humic acid was extracted from leonardite, the growth parameter, shoot, and dry weight of wheat increased with an increase in the nutrient content of the plant [15]. These results have strongly supported the hypothesis that the beneficial effect of humic and fulvic acid on plant development may be dependent on their capacity to improve nutrient availability for plant uptake under nutrient-deficient conditions. Various scientists reported that humic and fulvic acids increased the concentration of all the reliable and available nutrients uptake [16], while Verlinden *et al.* observed that the nutrient content of Maize, potato, and spinach crops significantly increased by humic substance [17]. The chemical and biological content available in FA or Humic acid increased the cation exchange and nutrient uptake [14].

Food production, distribution, and food security were set as primary national goals of the world; however, increasing population, climate change problems, and fewer production techniques reduce the economic development and agricultural production in China and other countries of the world [18]. To increase grain production and to fulfill, the population requirement, the application of FA began to apply to achieve the nutritional requirements, increase the plant growth characteristics, and nutrient uptake of Wheat-Maize crops.

This study was focused on the influence of FA derived from different materials on physicochemical characteristics, plant growth, and nutrient uptake of Maize-wheat production. We hypothesized that many researchers suggest humic substances (HA/FA) significantly increase the physical, and chemical properties of soils as well as physiological parameters and nutrient uptake of plants. FA of different Varieties such as Solid (S), Liquid (NL), and plant-derived Liquid (P) were used as treatment were obtained from Shandong Quan Linjia fertilizer Co. Ltd (Shandong, China) applied different concentrations on the maize-wheat crop grown on Albic (AL), Irrigated Desert (IR) and Shahjiang black (SH) soils to determine responses of various plant growth parameters (stem diameter, spike weight, plant height, biomass, and grain weight) and uptake of N, P and K.

The purposes of this study are (1) to evaluate the impact of fulvic acids (FA) obtained from different parent materials on the physiological characteristics of Wheat and Maize crops. (2) To evaluate the application of FA on low productive soils to enhance plant growth performance. (3) To evaluate and differentiate the FA performance on best-cultivated soil.

2. MATERIAL AND METHODS

2.1 Experimental soils

Three different soils albic black (AL), irrigated desert (IR), and Shahjiang black (SH) soils are classified as Udic, Aridisols, and Podzoluvisols soils according to the soil taxonomy of China (Soil

Survey Staff, 2014), were collected at the depth of 0-20 cm. The Albic black soil (AL) was sampled from Qiqihar city; district Jianhua (47°21′ N, 123°55′ E) located in the west part of Heilongjiang province, however, irrigated desert soil (IR) was sampled from Roughua village Liangzhou District, Wuwei city (37°55′ N, 102°38′ E) Gansu province and Shahjiang black soil (SH) was taken from demonstration base of Anhui Agriculture university Wanbei comprehensive test station (33°50′ N, 117°16′ E) Anhui province China. The soil samples were shipped to the soil fertility and improvement laboratory institute of environment and sustainable development in agriculture (IEDA). The pot experiment was situated at the institute of environment and sustainable development in agriculture (IDEA, CAAS), experimental farm, which was allocated at (40°09′N, 116°92′E) Shunyi District, Beijing China.

2.2 Basic Properties of Soil

The basic soil properties, such as soil EC, pH, Soil organic matter, total nitrogen, total phosphorus, and total potassium content of the three sites are different. The AL soil had the lowest soil EC and pH (30.1 μs cm^{-1} and 5.26), however, irrigated desert soil (IR) had the lowest soil organic matter (2.2 g kg^{-1}), cation exchange capacity (CEC) (4.6 cmol kg^{-1}) and total nitrogen (0.20 g kg^{-1}) as compared with the AL and SH soils. Similarly, it was observed that SH soil has the highest organic matter, CEC, and total nitrogen content. Other soil properties such as available nitrogen content was highest in AL followed by SH soil, available phosphorus was observed lowest in between the soil. However, total phosphorus (TP) and total potassium (TK) content was not much different between these three soils, although due to the change of parent materials between the soils the silt, sand, and clay content was also changed in which AL soil having a large amount of silt particles and having silty clay texture, however, IR soil was sandy loam in nature and SH soil was sticky when wet because of high clay content and the texture was clayey loam (Table 1).

2.3 Experimental Design

The experiment was conducted at Shunyi, an experimental area of the Institute of Environment and Sustainable Development, Chinese Academy of Agriculture Sciences (CAAS), Beijing, China. FAs were applied at four different concentration 0 (CK), 0.25 % (S), 0.50 % (NL), and 0.50 % (P) treatments with four replications of each treatment. The soil was ground and passed through a 5 mm sieve to remove gravel. The size of the pots was 30 x 50 cm. Plant-derived Solid (S) FA was mixed with 15 kg of soil in the pot. However, the liquid form was applied at first irrigation in a 27 and 29.5 cm diameter and height respectively in the pot.

Maize seeds were sown in summer, similarly, the wheat was sown in the winter season, respectively. A compound fertilizer with a composition of 25 % N, 14 % P_2O_5, and 7 % K_2O, was applied to a total of 15 g pot^{-1} (5 g at sowing, 5 g after transplanting, and 5 g at the maturation stage). Plants were harvested at the mature stage and dried at 65 °C. Each harvested plant part such as leaves, stems, and grains were separated according to the treatments and labeled carefully for the assessment of agronomic parameters of plant height, stem diameter, spike height, total plant biomass, thousand-grain weight, and plant nutrient uptake.

2.4 Elemental Composition of Fulvic acids

The soil-applied fulvic acid (FA) was obtained from Shandong Quan Linjia fertilizer Co. Ltd (Shandong, China). Chemical compositions of the three fulvic acids (FA) are presented in (Table 2).

2.5 Statistical Analysis

The collected data was subjected to One-Way ANOVA by using SPSS software 21.0 (SPSS, Version 21.0, Chicago, IL, USA). The multiple comparisons of means were done by using Tukey's HSD post hoc test. The data are expressed as the mean (n=4) ± SE (standard errors) and multiple comparison tests were performed at a significance level of < 0.05. However, graphs were prepared by using GraphPad Prism 6.

3. RESULTS

3.1 Influence of FA on Maize Diameter and Wheat Spike Weight

Results showed that the application of FA derived

Table 1. Properties of soils used in the experiment (mean ± standard error; n=3).

Soil	EC us/cm	pH	OM g/kg	CEC cmol/kg	AN mg/kg	AP mg/kg	AK mg/kg	TN g/kg	TP g/kg	TK g/kg
AL	30.1	5.26	8.4	21.6	66	0.44	78.0	0.70	0.39	20.4
IR	2063	8.49	2.2	4.6	19	2.4	525.3	0.20	0.39	20.1
SH	132	7.99	11.7	25.2	52	1.19	186.6	0.83	0.39	17.3

*Al (Albic black), IR (Irrigated desert) and SH (Shahjiang black), OM (organic matter), CEC (cation exchange capacity), AN (available nitrogen), AP (available phosphorus), AK (available potassium), TN (total nitrogen), TP (total phosphorus) and TK (total potassium)

Table 2. Elemental compositions of plant-derived solid (S), mineral-derived liquid (NL), and plant-derived liquid (P) fulvic acids.

FA Type	N	C	H	S
		%		
S	5.39	25.31	5.75	8.47
NL	10.29	52.476	9.74	14.84
P	10.78	50.61	11.56	16.96

S (Plant-derived solid), NL (Natural-derived liquid), and P (Plant-derived liquid) fulvic acids.

Table 3. Influence of FA on maize diameter and wheat spike weight

FA (%)	Stem Diameter mm (Maize)			Spike Weight g/pot (Wheat)		
	AL	IR	SH	AL	IR	SH
CK	24.91±1.43^{a}	14.40±0.88^{ab}	25.0±0.60^{a}	99.0±6.55^{b}	76.72±5.57^{ab}	132.4±8.23^{a}
0.25 S	12.92±1.46^{b}	15.68±1.16^{a}	26.16±0.76^{a}	123.24±6.63^{a}	30.3±5.50^{b}	124.2±8.85^{ab}
0.50 NL	24.95±12.2^{a}	12.27±1.33^{b}	26.90±1.05^{a}	97.25±4.63^{b}	87.14±5.73^{a}	126.8±5.23^{ab}
0.50 P	26.35±0.56^{a}	18.16±2.37^{a}	25.77±1.0^{a}	68.51±8.20^{ab}	98.51±9.21^{ab}	76.75±8.45^{b}

*Different letters (a, b) show significantly different at ($p<0.05$). Standards are mean ± standard error, (n= 4). Plant-derived Solid (S), Natural-derived Liquid (NL), and Plant-derived liquid (P) on Albic Black (AL), Irrigated Desert (IR) and Shahjiang Black (SH) soils.

from different parent materials significantly increased ($p < 0.05$) stem diameter and spike weight in three soils; AL, IR, and SH (Table 3). In AL, the application of FA significantly increased the stem diameter of maize by 24.9 mm as compared to control (no FA) to 24.95 and 26.3 mm at NL and P, similarly, 14.4 mm in control to 15.6 and 18.16 mm in IR and 25 mm in control, 26.1, 26.9 and 25.7 in SH soils, however, decreased 12.9 and 12.2 mm in AL and IR soils with the application of 0.25 % S, 0.50 % NL and 0.50 % P respectively (Table 3). The results observed an increase of 0.16 % and 5.62 %, 8.33 % and 26.11 % and 4.58 %, 7.6 % and 3.08 % among the treatments, in AL, IR, and SH soil, respectively. However spike weight of wheat was decreased by 48 % in AL, and 15 % in IR soil at 0.25 % S and 0.50 % NL treatments respectively. On an average basis, it increased when compared to control by 3 %, 17 %, and 14 % among the treatments at AL, IR, and SH soils respectively. It is evident from this study that a higher response of FA was observed on SH soil vis-a-vis AL and IR soils which could be ascribed to improvement in soil physical properties. Application of FA increased 123.2 g pot^{-1}, 87.1 g pot^{-1}, and 98.5 g pot^{-1} in AL and IR soil among the treatments. However, decreased 97.2, 68.5 g pot^{-1} in AL, 30.3 g pot^{-1} in IR, and 124.2, 126.8, and 76.5 g pot^{-1} in SH soils among the treatments respectively. Showing an increase of 24 %, 12 %, and 24 % in AL and IR soils respectively. The average increase among the treatments was 24 % in AL, 21 % in IR, and decreased 17 % in SH

soils over control.

3.2 Influence of FA on Plant height and Biomass of Maize-Wheat

Plant height and biomass content were significantly and non-significantly increased ($p < 0.05$), with different types, and levels of FA after harvesting of maize and wheat at AL, IR, and SH soils (Figure 1). There was an increase of plant height over the control of 3 % and 5 % in AL, 9 % and 11 % in IR, and 1 %, 11 %, and 1 % in SH soils among the treatments after harvesting of maize, however, decreased 42 % and14 % at 0.25 % S and 0.50 % NL in AL and IR soils respectively (Figure 1 A). An overall, results showed an increase of 8 % and 9 % over control on 0.25 % S, 3 % and 11 % at 0.50 % NL and 5 % and 11 % on 0.50 % P treatments in three soils. Similarly, an increase in the plant height of wheat over control of 4 % and 1 % in AL and 5 % in IR soil and decreased 3 %, 16 % and 12 %, 5 %, 7 %, and 6 % in AL, IR and SH soils respectively among the treatments (Figure 1B). On the other hand, an increase of biomass after maize harvesting over control (no FA) was 38 %, 12 % and 41 %, 23 %, 34 %, and 17 % on AL, IR, and SH soils respectively among the treatments, however, decrease of 74 %, 5 % and 17 % at AL and IR soils among the treatments (Figure 1C). Similarly, biomass content of wheat increased over control by 21 %, 12 %, and 29 % in AL, 8 % and 5 % in IR, and 22 % in SH soil among the treatments, however, decreased by 46 %, 14 %, and 3 % in IR and SH soils on 0.25 % S and 0.50 % P treatments respectively (Figure 1D).

3.3 Influence of FA on Thousand Grain Weight of Maize-Wheat

Grain weight was significantly and non-significantly increased ($p < 0.05$), with different types, and levels of FA after harvesting of maize and wheat at AL, IR, and SH soils (Figure 2). There was an increase in grain weight over the control of 15 % in IR and

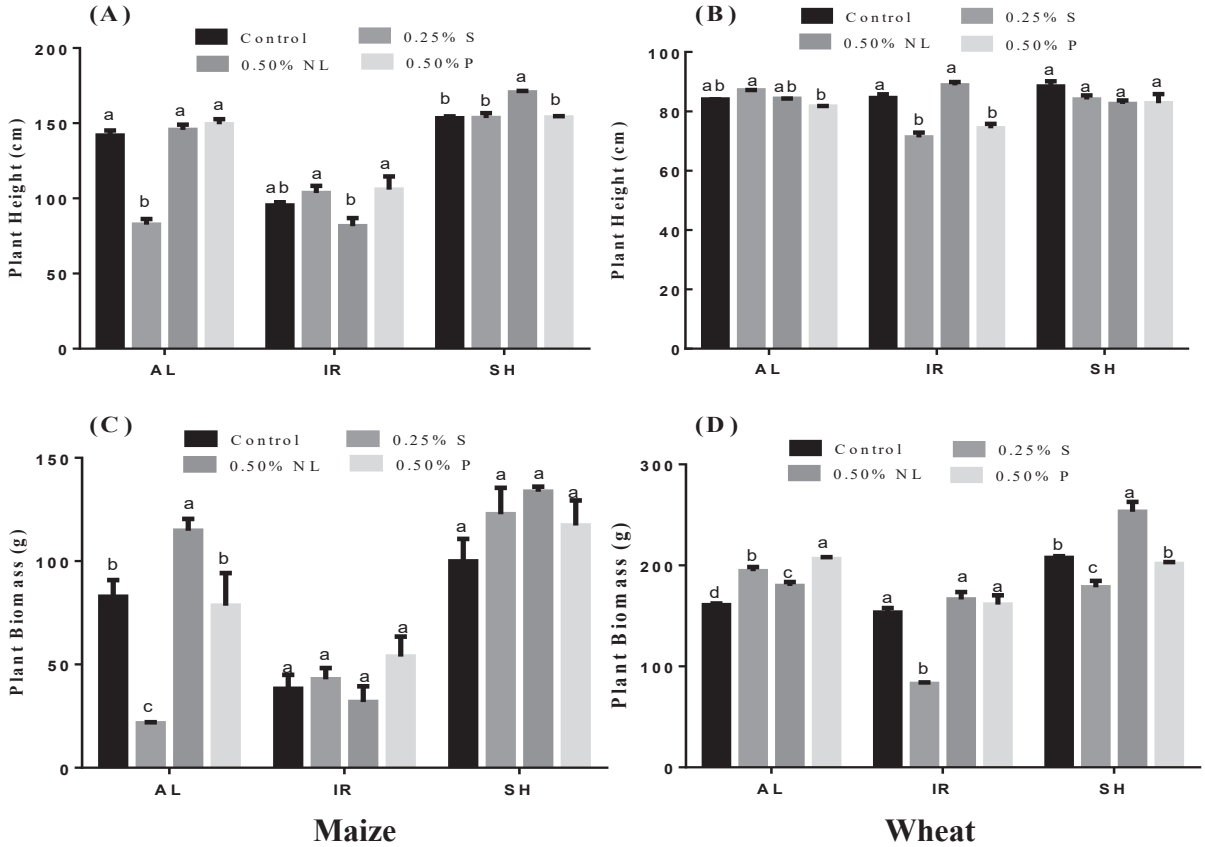

Fig. 1. Influence of 0.25 % S, 0.50 % NL and 0.50 % P FA on Plant height and Biomass on (A) Maize Plant height, (B) Wheat Plant height, (C) Maize Biomass and (D) Wheat Biomass content of dry soil from pot experiment on AL, IR and SH soils. Letters on top show the difference among the treatments, the error bar shows the standard errors of the mean (n=4) with probability (LSD 0.05) at 5 %.

14 %, 34 %, and 2 % in SH soils after harvesting of maize, however, decreased by 46 %, 20 %, and 1 % in AL, 9 % and 8.5 % in IR soils respectively among the treatments (Figure 2). Similarly, the grain weight content of wheat showed a significantly decreased from control by 2 %, 6 %, and 5 % in AL, 16 %, 8 %, and 15 % in IR, and 11 %, 13 %, and 6 % in SH soils among the treatments (Figure 2). Overall, results showed that grain weight content there was an increase in maize grain weight around 15 % in IR soil and 3 % to 34 % in SH soils, however, wheat grain weight significantly decreased and was found lower concentration than maize grain weight.

3.4 Influence of FA on Nutrients Uptake of Maize

Nutrient uptake (N, P, and K) content of maize was significantly and non-significantly increased ($p < 0.05$), with different types, and levels of FA after harvesting of maize at AL, IR, and SH soils (Figure 3). There was an increase of nitrogen over control of 21 %, 34 %, and 52 % in AL, 24 % and 3 % in IR, and 5 % in SH soils among the treatments after harvesting of maize, however, decreased by 14 %, 2 % and 3 % at 0.25 % S and 0.50 % P in IR and SH soils respectively (Figure 3A). Similarly, an increase in the phosphorus uptake over control of 50 % and 16 % in AL, 5 % and 3 % in IR, and 4 %, 1 %, and 5 % in SH soils and decreased 45 %, 14 % in AL, IR soils respectively on 0.50 % P treatment (Figure 3B). On the other hand, potassium uptake increased over control (no FA) was 17 %, 2 %, and 3 % in AL, 6 %, 7 %, and 12 % in IR, and 3%, 4%, and 54% in SH soils among the treatments (Figure 3C). Overall, results showed that Nitrogen uptake was increased by 21 % to 52 % over control in AL, 20 % to 24 % in IR, and 4 % to 5 % in SH soils, however, phosphorus was increased by 16 % to 50 %, 3 % to 5 % and 4 % to 5 % in AL, IR, and SH soil respectively. In the case of potassium, uptake results showed that potassium was highly uptake and increased by 3 % to 17 %, 7 % to 12 %, and 4 % to 54 % in SH soils respectively among the treatments as compared with control (Figure 3).

3.5 Influence of FA on Nutrients Uptake of Wheat

N, P, and K uptake of wheat was significantly and non-significantly increased ($p < 0.05$), with different types, and levels of FA after harvesting of the wheat at AL, IR, and SH soils (Figure 4). There was an increase of 37 % and 9 % in AL, 6 % in IR soils among the treatments as compared with control, however, decrease of 17 %, 12 %, 29 %, 34 %, 47 %, and 32 % among the treatments in AL, IR and SH soils respectively (Figure 4A). Similarly,

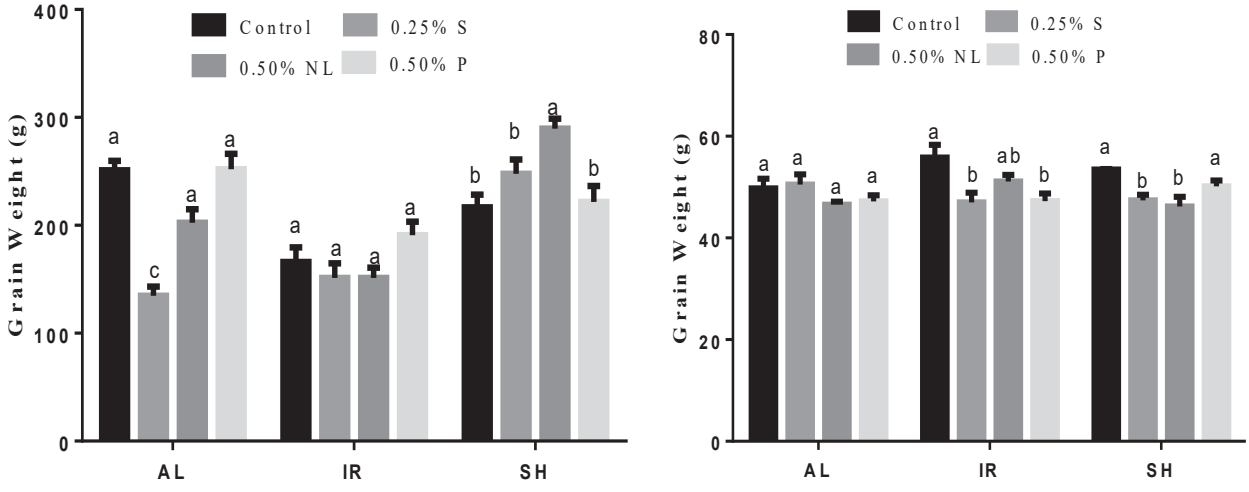

Fig. 2. Influence of 0.25 % S, 0.50 % NL and 0.50 % P FA on Grain weight content of Maize-wheat of dry soil from pot experiment on AL, IR, and SH soils. Letters on top show the difference among the treatments, the error bar shows the standard errors of the mean (n=4) with probability (LSD 0.05) at 5 %.

an increase in the phosphorus uptake over control of 10 % in AL, 8 %, and 2 % in IR and decreased 12 %, and 6 % in AL, 6 % in IR, and 22 %, 7 %, and 12 % in SH soils respectively among the treatments as compared with control (Figure 4B). On the other hand, potassium uptake increase over control (no FA) was 9 % and 8 % in AL, 36 %, 4 %, and 30 % in IR, and 1 % in SH soils among the treatments and decreased by 9 %, 8 % and 1 % among the treatments at AL and SH soils respectively (Figure 4C). Overall, results showed that nitrogen uptake was increased by 22 % over control in AL, and 5 % in IR soil; however, phosphorus was increased by 10 % and 5 % in AL and IR soils respectively. It was also recorded that nitrogen and phosphorus uptake was highly decreased in SH soil. In the case of potassium, uptake results showed that potassium was increased by 9 %, and 23 % in AL and IR soils respectively, similarly, potassium uptake in SH soil was decreased among the treatments as compared with control (Figure 4).

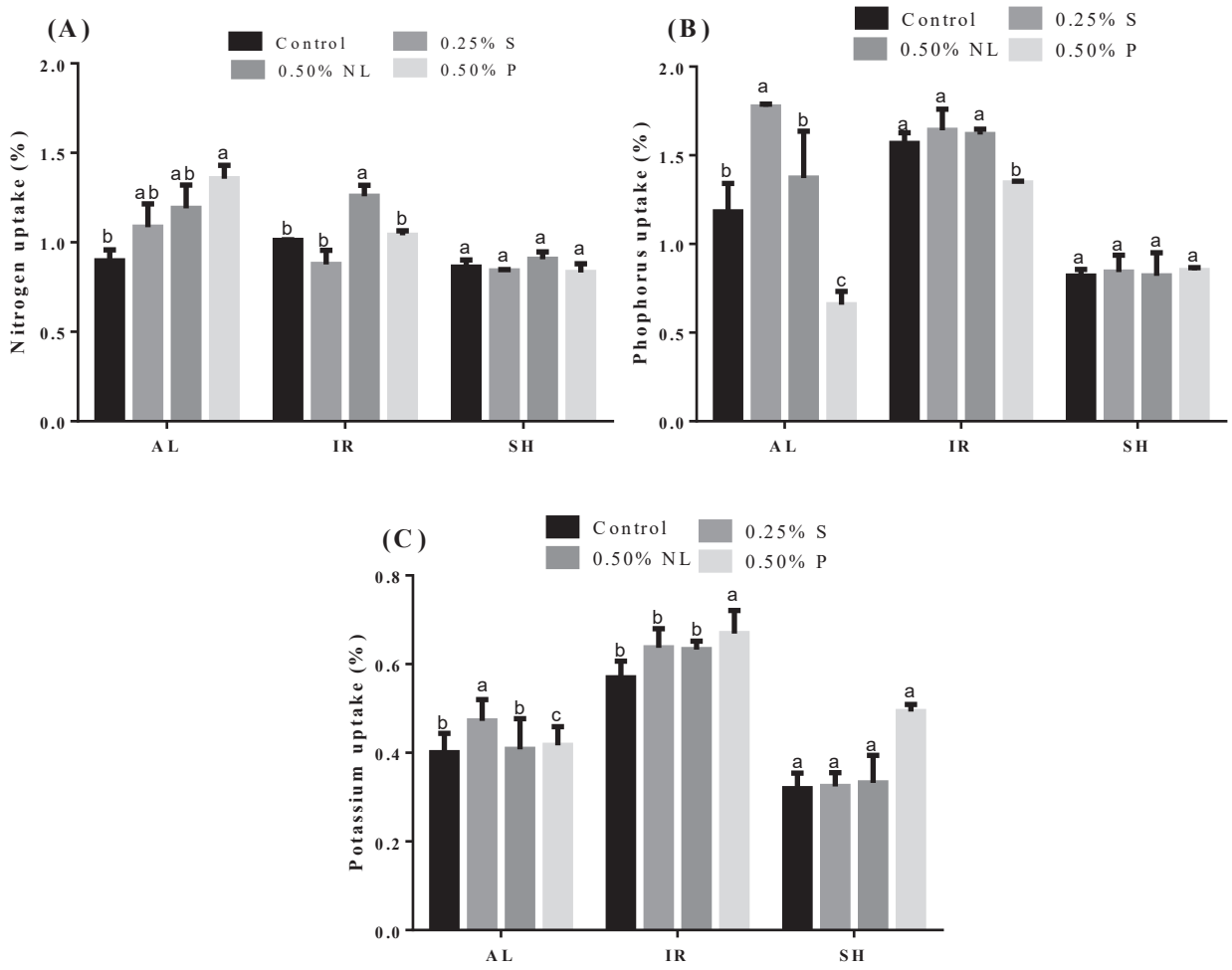

Fig. 3. Influence of 0.25 % S, 0.50 % NL and 0.50 % P FA on Nutrient uptake of Maize of dry soil from pot experiment on AL, IR, and SH soils. Letters on top show the difference among the treatments, the error bar shows the standard errors of the mean (n=4) with probability (LSD 0.05) at 5 %.

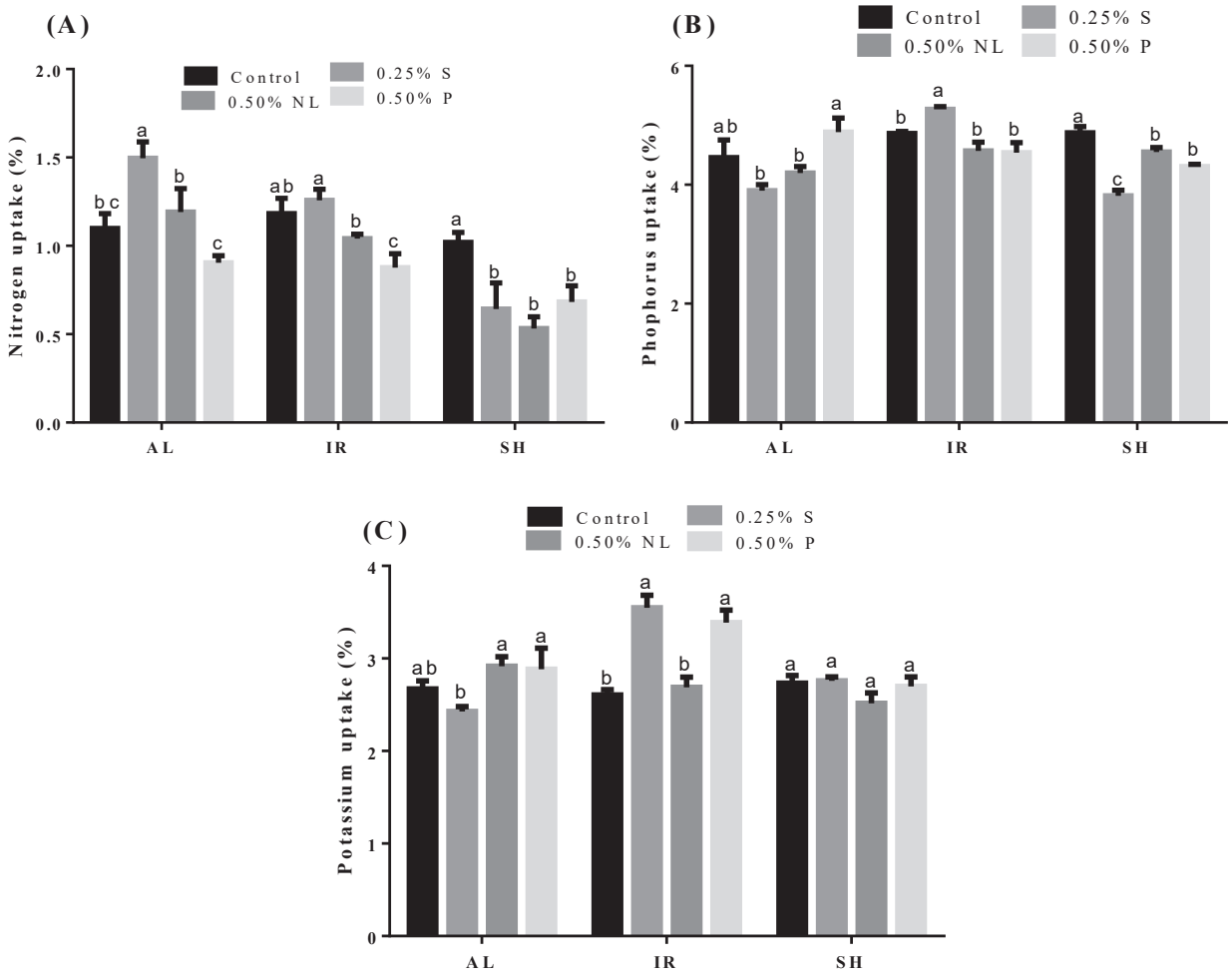

Fig. 4. Influence of 0.25 % S, 0.50 % NL and 0.50 % P FA on Nutrient uptake of Wheat of dry soil from pot experiment on AL, IR, and SH soils. Letters on top show the difference among the treatments, the error bar shows the standard errors of the mean (n=4) with probability (LSD 0.05) at 5 %.

4. DISCUSSION

4.1 Effect of FA on Growth Parameters of Maize-Wheat

Fulvic acid (FA) is a natural product, that improved the physicochemical and biological properties of the soils [19], FA can also ameliorate the soil conditions and bring evenness throughout the plant [2, 20], plant residues are the upright cause of plant nutrients and the vital mechanisms for the constancy of agricultural ecosystems. FA organically improvement of soil increased the yields of some field crops in several studies. Our results indicated that the application of FA significantly increased the stem diameter content of maize on AL and IR soil, and non-significantly increased it in SH soil. It was also observed that application 0.25 % S FA was found lower as compared with the other two FAs. Similarly, the spike weight of wheat was also increased in which the highest spike weight was observed in SH soil as compared with AL and IR soils (Table 3). The increase in stem diameter and spike weight may be due to the positive effect of FA on growth parameters and maybe due to the nutrients supplied by these amendments [21]. FA may augment the plant growth characteristics, nutrient uptake and reduce the perception of harmful components and improve plant metabolism [22]. Our results showed plant growth and biomass content of maize-wheat on AL, IR, and SH soils were increased after FA treatments, however, reduces at 0.25 % S treatment in maize crop. Similarly, it was observed that FA treatments had a better impact on wheat growth parameters as compared with maize (Figure 1). The increase in the growth parameters

proves that supplement of HA/FA in soil increased the growth characteristics of plants. The reduction in growth parameters due to 0.25 % S could be because Solid FA was applied in a small amount. There could be another justification which may be because FA in powder form was not dissolved entirely in the soil solution, however the liquid FA mix-up with the soil solution. The plant height of the Petunia hybrid 'Dream Neon Rose' increased with humic acid treatments as indicated by Chamani *et al.* [23]. However, Khaled and Fawy [24] observed that wheat growth was significantly increased by the application of FA and HA weather mixed in soil or with foliar application. Similarly, Sharif *et al.* [14] reported that when a low concentration of FA compared with control in soil significantly increased maize yield, similarly, the higher concentration of more than 300 mg kg^{-1} reduced the yield. FA and HA also increased the yield and yield component of wheat and different grain-producing crops i.e. 1000-grain weight, biological yield, dry matter, grain yield, and harvest index were significantly affected by HA Qin *et al.* and Tuba *et al.* [25, 26]. The present study also indicates that the 1000-grain weight of maize and wheat was increased by the application of FA on AL, IR, and SH soils, the average highest grain weight was recorded in SH soil on the 0.50 % P treatment in Maize crop, however, there was the non-significant difference was observed between the treatment of IR soils as the grain weight was also found lower. Similarly, the 1000-grain weight of wheat was found quite similar in each treatment and a non-significant difference was observed on AL soil (4.2). The results in yield and yield components that were observed in this study was supported by previous findings of Tahir *et al.* [13]. These findings were previously reported by Sharif *et al.* [14] who suggested that the grain yield of maize was recorded higher at low concentrations and observed a significant decline in the yield and non-significantly reduces the grain yield at a higher dose of HA. Similarly, Zhang *et al.* [2] found that it increases the grain yield of legumes such as mung bean (mash bean=moong) (*Vigna radiata* L.), soybean (*Glycine max* L.), and pea (*Pisum sativum* L.). The enhancement of plant growth characteristics of maize and wheat with the addition of FA could be due to the presence of carboxyl and hydroxyl groups and growth-promoting substances such as gibberellins indole acetic acid which may have straight involvement in cell respiration, photosynthesis oxidative enzymatic reactions [16]. The enhancement of root development through FA was outstanding. It was reported by Shahryari *et al.* and Saruhan *et al.* [27, 20] that the root hairs and its development through FA enhanced the plant's physiological characteristics by increasing the nutrient uptake in soil [13].

4.2 Influence of FA on Nutrient uptake of Maize-Wheat

The beneficial effect of humic and fulvic acids on plant development may be dependent on their capacity to improve nutrient availability for plant uptake under nutrient-deficient conditions. Various scientists reported that humic and fulvic acids increased the N, P, and K uptake [16], whereas Verlinden *et al.* [17] have observed that the HA or FA significantly increased the nutrient uptake in maize, wheat, and other vegetable crops. Regarding the above facts, our results indicated that nitrogen uptake in maize was increased by FA treatment and the highest nitrogen uptake was observed in AL soil on 0.50 % P treatment, however, non-significantly decreased on SH soils. Similarly, phosphorus uptake was also significantly increased between treatments on each soil highest observation was recorded at IR soil between the treatments as compared with AL and SH soils. The potassium uptake of maize was increased among the treatments but found non-significant on SH soil (Figure 4). It was also observed that the N, P, and K uptake of wheat was also increased by the application of FA on AL, IR, and SH soils. Nitrogen uptake of wheat was increased between the treatment but decreased on SH soils, the highest N uptake was recorded on 0.25 % S treatment on AL soil as compared with IR and SH soil (figure 4A). However, the uptake of Phosphorus and potassium was increased among the treatments on AL and IR soils and decreased on SH soil. The highest phosphorus and potassium uptake was recorded on 0.25 % S treatment in IR soil (figure 4B & C). FA accelerates the nutrient composition in wheat and maize could be because FA and HS substances are filled with microbiological and other elements [28], and enhance nutrient uptake [29]. Results also correspond with Nikbakht *et al.* [16] studied that the macro and micronutrients content of Gebra leaves was increased significantly when HA was spread at 1g L^{-1}. Similarly, HA increased

the nutrient concentration and growth of maize plants even in saline conditions [24]. They have also observed that HA mixed with soil increased N-uptake, whereas foliar application significantly enhanced the other nutrients uptake such as (P, K, Mg, Ca, Zn and Cu). Similarly, HA and straw-derived fulvic acid application also increased the nutrient uptake of watermelon leaves and tomatoes in hydroponic culture and field soils [2, 30, 31].

5. CONCLUSION

FA application increased the growth characteristics of the plant and improves the quality and quantity of fruits crops through the carboxyl and hydroxyl mechanisms which are involved in cell respiration, photosynthesis, water, and nutrient uptake, and enzyme activities. The stimulating effects of FA have been directly correlated with enhanced uptake of macronutrients, such as nitrogen, phosphorus, and sulfur, and micronutrients like Fe, Zn, Cu, and Mn. Due to a decline in the soil's chemical characteristics and reduction in plant growth performance and crop production. To increase crop production and increase the capability of soil characteristics, the plant growth characteristics and plant nutrient uptakes were determined. Our results showed that the application of FA increased the Plant height, biomass, and 1000-grain weight. Besides increasing production, FA also increases the nutrient uptake in plants. It was also observed that application of 0.50 % P FA increased the plant growth characteristics and nutrient uptake of the maize-wheat crop in AL soil, however, application of 0.50 % NL FA increased the plant characteristics of the maize-wheat crop in IR and SH soil. Overall, we found that FA application in liquid forms performs better than the solid FA in IR and SH soils as compared with AL in which 0.50 % P shows better improvement in crop growth in each mentioned soil. It is suggested that to increase the physiological parameters and increase the yield component FA in a Liquid state should be applied in field conditions, and consider the economic level to improve the management strategies

6. CONFLICT OF INTEREST

The authors declare no conflict of interest

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Research Article

Frequency of Different Types of Endothelial Corneal Dystrophies by Age, Gender, and Visual Acuity in Punjab, Pakistan

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Abstract: Endothelial Corneal Dystrophy (ECD) is, by definition, an endogenous degeneration that progresses slowly in the corneal endothelium as a result of genetic predisposition. A transverse study was conducted from September 2018 to June 2019, and the subjects affected with ECD were examined by visiting various city hospitals in Punjab including Sahiwal, Narowal, Okara, Gujranwala, Kasur, Lahore, and Multan. The basic objective of the current investigation was to find the frequency of different types of ECD in the population of different cities in Punjab. Data was collected based on relevant parameters such as age, gender, and visual acuity. The visual acuity was assessed by ophthalmologists via Snellen chart and Visual acuity test. Among 3000 patients, 6.6 % (n=198) cases of ECD were recognized which includes more males 52.52 % (n=104) as compared to females 47.48 % (n=94). Maximum cases of ECD 31.82 % (n=63) were observed in the 41-50 years of age group. In all types of ECD, FECD was observed most abundant with 38.38 % (n=76) and XECD with the least abundance of 6.67 % (n=13). The frequency of CHED and PPCD was 25.25 % (n=50) and 29.79 % (n=59) respectively. The results of recent research finalize that endothelial corneal dystrophy is a rare disease prevailing in Pakistan with a proportion of only 6.6 %. This study benefits in updating the data about the frequency of endothelial corneal dystrophy in Pakistan. Careful clinical evaluation, initial diagnosis, genetic counseling, genotyping, and correct treatment are necessary for the restoration of vision loss due to ECD.

Keywords: Endothelial Corneal Dystrophy, Frequency, Fuchs Endothelial Corneal dystrophy, Visual acuity, Cornea, Visual impairment

1. INTRODUCTION

The visual system constructs a mental representation of the world around us. The cornea is a dome-shaped front portion of the eye where light is focused [1]. In ophthalmology, corneal dystrophies (CD) are characterized as a group of bilateral, symmetrical inherited disorders that progress slowly with no systemic or environmental factors [2]. Corneal dystrophies can be classified as Phenotypic approaches such as "anatomic-histologic", classification based on corneal layers (epithelial layer, epithelial-stromal layer, and stromal layer or endothelial-descemet layer) and another is "anatomic-descriptive" that is based on appearance, which appears in the affected corneal layers (granular, lattice, mosaic, amyloid, etc.) and Genetic approaches, combine Mendelian genetics employing molecular genetics [3]. World Health Organization (WHO) categorizes vision loss into three classes, low vision i.e., $6/60 \leq VA < 6/18$; $10^{o} \leq VF < 20^{o}$, severe vision impairment i.e., $3/60 \leq VA < 6/60$; $5^{o} \leq VF < 10^{o}$, and profound vision impairment i.e., $VA < 3/60$; $VF < 5^{o}$, which are considered to be authentic [4]. The significant causes of blindness are corneal dystrophy which affects at least 4 million people worldwide. The major cause of blindness in these children is CD

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and nearly 1.5 million children are blind worldwide [5]. The predominance of CDs varies between different parts of the world [6]. The prevalence of CD is 13.9 % in Europe. In Japan and Australia, 17 studies were conducted which revealed frequencies of CD 12.6 % and 07 % respectively. India found a 9.6 % prevalence of CD [7]. The prevalence of CD is like 897 per million and translates it to around 278,000 individuals in individuals of 310 million in the USA [5] and 60 % are of endothelial origin [3]. In one descriptive study conducted in Islamabad, out of 63 patients 12 were diagnosed as CD [7]. In Pakistan, the occurrence of blindness is 0.9 %. Corneal blindness is the leading cause of blindness nationally afterward cataracts which also adds 11.8 % of the total blindness in Pakistan [8]. The predominant cause of corneal opacity was considered decreased visual acuity [9].

ECD is characterized as endogenous erosion of the endothelial layer of the cornea due to genetic dispositioning. It is a slow-acting disease and in most of them, the endothelium layer transports a defective active fluid causing disproportionate edema of the corneal stroma, which damages the corneal clarity and lessens the visual acuity of the affected individual [10]. Generally, CD patients may appear to be asymptomatic or may complain about blurred vision [11]. Fuchs endothelial corneal dystrophy (FECD) with drop-like conglomerates or guttae and Descemet's membrane [12] is highly prevalent amongst the corneal dystrophies [13]. In 2012, 39 % of corneal transplants were to treat FECD worldwide [14]. The occurrence of FECD is concluded as 1/2000 in females. The prevalence of over 40 years of age is about 4–11 % [15]. Posterior Polymorphism Corneal Dystrophy (PPCD) including vesicles, breaking bands, or gray haze [16] happens to affect 1/100,000 individuals in the population [3]. In Congenital Hereditary Endothelial Dystrophy (CHED) there is the appearance of edema in the affected layer along with diffuse ground glass-like corneal haze and focal gray spotting leading to blurred vision [17]. X-linked Endothelial Corneal Dystrophy (XECD) symptomize as moon crater-like endothelial cells in addition to diffuse milky opacities with band keratopathy [18].

The current study aimed to determine the frequency of different types of ECD by age in males and females and the association of visual acuity with endothelial corneal dystrophies in various cities of Punjab, Pakistan. The impact of ECD involves the deterioration of corneal transparency, potentially resulting in vision loss or blurriness. So, most specifically this study would provide awareness to the patients regarding disease and their families for the reduction of disease burden and also a way for proper investigation of the disease.

2. MATERIAL AND METHODS

Individuals who were affected with ECD were identified by visiting different hospitals of different cities of Punjab i.e., Sahiwal, Narowal, Gujranwala, Okara, Kasur, Lahore, and Multan. Institutional Review Board (IRB) approval was obtained from the ethical committee of Lahore College for Women University, Lahore, Pakistan along with the permission from the administration of selected hospitals was also obtained to collect data to review the records and examine patients with help of expert ophthalmologists to fulfill the inclusion criteria. We excluded all other types of corneal dystrophies (i.e. Epithelial and Stromal corneal dystrophies and their subtypes). Proforma was designed to collect initial data including address, analysis, and status of the disease from the patients at hospitals. The records of the corneal patients were also noted from the hospitals with the assistance of expert doctors and staff to complete information about ECD patients. Ophthalmologists used various methods such as the Snellen chart to test visual acuity as a fraction, Pachymetry to calculate the thickness of the cornea, and the Keratometer to calculate the curvature of the epithelial surface of the cornea, and Topography to convert surface curvature to three-dimensional maps. Case files of patients with ECD dystrophies were also evaluated. The variables which were analyzed are:

- Gender
- Age
- Far visual acuity
- Main complaint (Endothelial corneal dystrophy)
- Any associated pathologies

Data collected was arranged and chi-square was used to evaluate variables and results were measured as significant at $P < 0.05$. The analyzed variables on which chi-square is applied included

age, gender, and visual acuity.

3. RESULTS AND DISCUSSION

A total of 6.6 % (n=198) of 3000 patients had ECD, with men accounting for 52.52 % (n=104) and females accounting for 47.48 % (n=94). In our study, 198 patients of ECD were observed in which the frequency of males 53.52 % (n=104) is more than females 47.48 % (n=94). For age distribution, we considered seven categories (0-10, 11-20, 21-30, 31-40, 41-50, 51-60, and 61-70 years)(table 1). The most frequent age group was between 41-50 years which comprised of 31.82 % (n=63) with 46.03 % (n=29) of males and 52.96 % (n=34) females. The age group 61-70 years had the fewest cases of ECD, accounting for 2.52 % (n=5) of the total, with 40 % (n=2) of men and 60 % (n=3) of females.

Among the eyes (bilateral) of 198 patients with known visual acuity, 76 were of FECD out of which 42 were males and 34 were females. In CHED, the eyes of 50 patients were identified of which 32 were males and 18 were females. In PPCD, the eyes of 59 patients were identified of which 25 were males and 34 were females. In XECD, the eyes of 13 patients were identified of which 8 were males and 5 were females.

3.1.1 Frequency of Fuchs Endothelial Corneal Dystrophies in patients by Age and Gender

Out of 198 cases of endothelial corneal dystrophies, 38.38 % (n=76) were of FECD from which 55.26 % (n=42) were males and 44.74 % (n=34) were females. Cases of FECD have only been observed in three age groups and the most prevalent group was 41-50 years with 57.89 % (n=44) cases among which 52.27 % (n=23) were males and 47.73 % (n=21) were females. On the other hand, the least group was 51-60 years with 5.26 % (n=4) cases and it was found to be 50 % (n=2) males and 50 % (n=2) females. In third age group 61-70years cases were 36.84 % (n=28) which included 60.71 % (n=17) males and 39.28 % (n=11) females. The predominance of visual disability in 76 patients of FECD concerning gender is explained in Fig. 2.

3.1.2 Frequency of Congenital Hereditary Endothelial Corneal Dystrophies by Age and Gender

Out of 198 cases of endothelial corneal dystrophies, 25.25 % (n=50) were of CHED from which 64 % (n=32) were of males and 36 % (n=18) were of females. In >10 age group, only 8 % (n=4) cases were observed in which 50 % (n= 2) were males and 50 % (n=2) were females. In 11- 20 years age group, among 20 % (n=10) cases, 80 % (n=8) were males and 20 % (n=2) were females. In the age of 21-30 years, there were 26 % (n=13) cases in which 69.23 % (n=9) were males and 30.77 % (n=4) were females.

In the age group of 31-40 years, 30 % (n=15) cases were examined among which 66.67 % females. In the age group 41-50 years, 16 % (n=8) cases were observed among which 62.5 % (n=5)

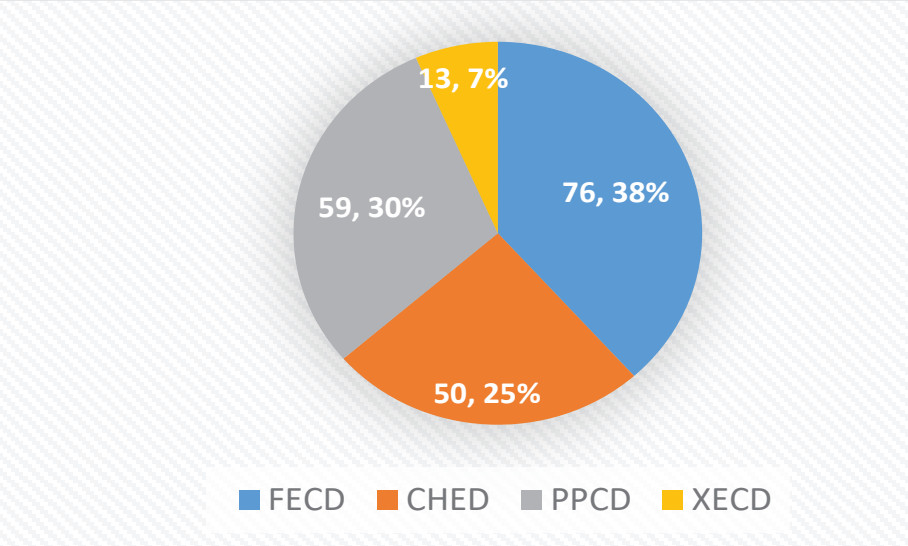

Fig. 1. Frequency of different types of endothelial corneal dystrophies in 198 patients

were females and 37.5 % (n=3) were males (Fig. 3).

3.1.3 Frequency of Posterior Polymorphous Endothelial Corneal Dystrophies by Age and Gender

There were 198 cases of endothelial corneal dystrophies, 29.79 % (n=59) were of PPCD from which 42.37 % (n=25) were of males and 57 % (n=34) were of females. In the age group >10 years, out of 8.47 % (n=5) cases, 80 % (n=4) were males and 20 % (n=1) female was observed. Among 22.03 % (n=13) cases, 8.47 % (n=5) males and 61.54 % (n=8) females were examined in 11-20 years of age group. A total of 33.89 % (n=20) cases were observed in 21-30 years of age group among which 40 % (n=8) were males and 60 % (n=12) were females. In 31-40 years, of age group, cases were 18.64 % (n=11) among which45.45 % (n=5) were males and 54.55 % (n=6) were females. 8.47 % (n=5) cases were observed in age group of 41-50 years and they were 100 % (n=5) females. In 51-60 years, age group, among 6.78 % (n=4) cases, 75 % (n=3) males and 25 % (n=1) female were observed while only 1.69 % (n=1) 100 % (n=1) female was observed in 61-70 years age group. More males than females were examined in PPCD (Fig. 4).

3.1.4 Frequency of X-Linked Endothelial Corneal Dystrophies by Age and Gender

There were 198 cases of endothelial corneal dystrophies. Out of which cases 6.57 % (n=13) were of XECD from which 61.5 % (n=8) were of males 38.5 % (n=5) were of females.7.69 %(n=1) of female was observed in the 10-20 years of age group. 7.69 % (n=1) of female was observed in the 21-30 years age group. In age group 31-40 years, there were 38.46 % (n=5) cases among which 40 % (n=2) were males and 60 % (n=3) were females. In age group of 41-50 years, among 46.15 % (n=6) cases, 50 % (n=3) were males and 50 % (n=3) were females. More females than males were examined in XECD (Fig. 5).

Corneal endothelial dystrophy is a hereditary, progressive, non-inflammatory, and bilateral disease affecting corneal transparency and refraction and leading to varying degrees of visual instability which involves dysfunction of the corneal endothelium layer. They include CHED, FECD, PPCD, and XECD and share many other characteristics including, corneal decomposition, distorted morphology of endothelial cells, and discharge of an irregular posterior collagenous layer in the posterior zone of Descemet's membrane, the endothelial basement membrane [19].

Relative findings with our recent data involve different research from different areas. In one descriptive study of the 63 patients, 12 (19 %) were diagnosed as having corneal dystrophies. In these 12 patients, 50 % (n=6) were diagnosed as stromal corneal dystrophies and 42 % (n=5) had posterior corneal dystrophies, and 8 % (n=1) had anterior corneal dystrophy [7]. In our study among 3000 patients, 6.6 % (n=198) cases of ECD were recognized which includes more males 52.52 % (n=104) as compared to females 47.48 % (n=94). In our study, 198 patients with endothelial corneal dystrophy were observed in which the frequency of males with 53.52 % (n=104) is more than females which are 47.48 % (n=94).

In our study out of 198 cases of endothelial corneal dystrophies, 38.38 % (n=76) were of FECD with a predominance of 55.26 % (n=42) of males as compared to females 44.74 % (n=34). In another study, FECD prevalence was 39.3 % in women and 30.8 % in men [20]. Twelve (or 19 %) of the 63 patients in the research were identified with corneal dystrophies. The average age was 31 ± 24.2 years, with about equal gender distribution. Males made up % of the group, while females made up 42 %. Six (50 %) of the 12 patients had stromal corneal dystrophies, five (42 %) had endothelial corneal dystrophies, and one (8 %) had anterior corneal dystrophy [21].

Of the 397 patient records reviewed and patients examined, 295 (178 women and 117 men) met our inclusion criteria. FECD prevalence among women was 39.3 % and 30.8 % among men. In men, prevalence increased for each increasing age group, from 6 % in 18-29-year olds to 69 % in 80-99 years old. The greatest increase (3.6-fold) happened amongst the age categories of 50-59 and 60-69 years. While the frequency of FECD increased by age in men, the frequency for women peaked in the 50-59 and 70-79-year age groups and was lower in other age groups. The greatest increase (4.2-fold) occurred between the age groups 30-49 and 50-59 [20]. In our study, out of 198 cases of endothelial

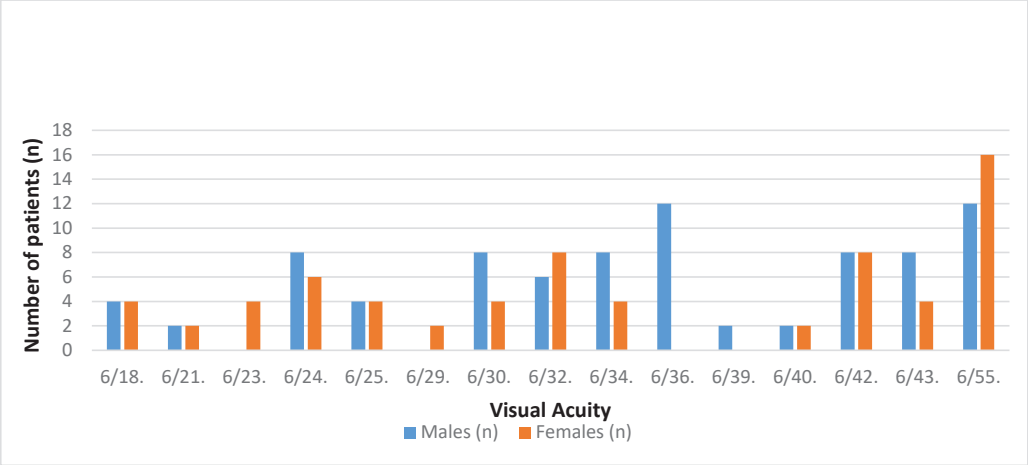

Fig. 2. The frequency of Visual Acuity in 152 eyes affected with FECD

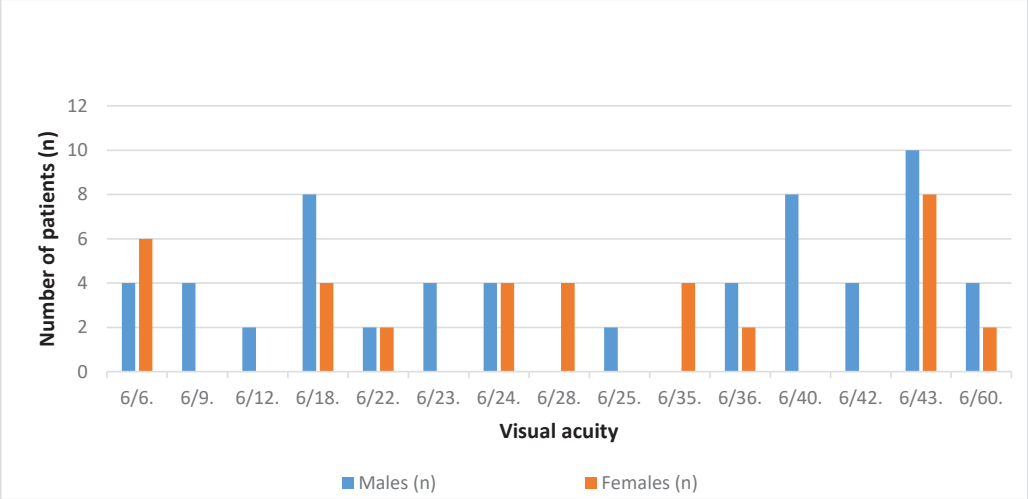

Fig. 3. The frequency of Visual Acuity in 100 eyes affected with CHED

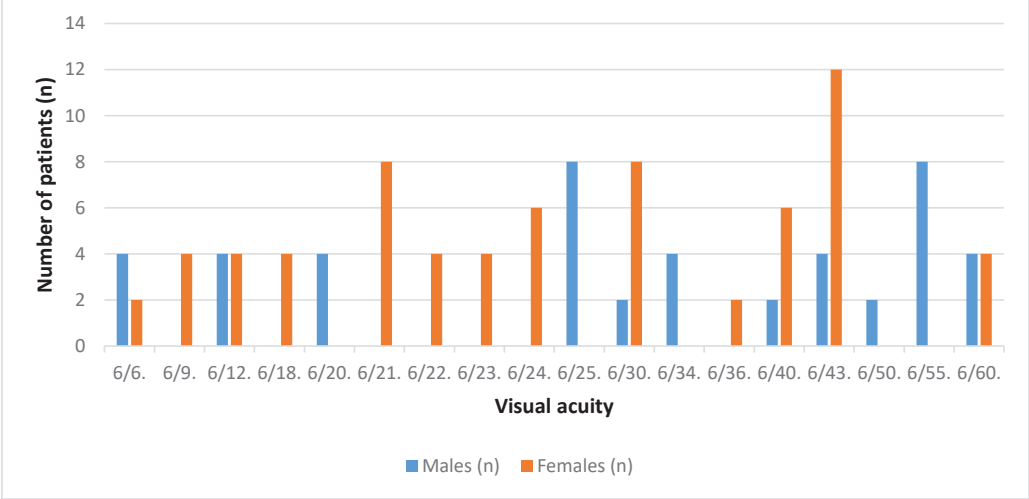

Fig. 4. The frequency of Visual Acuity in 118 eyes affected with PPCD

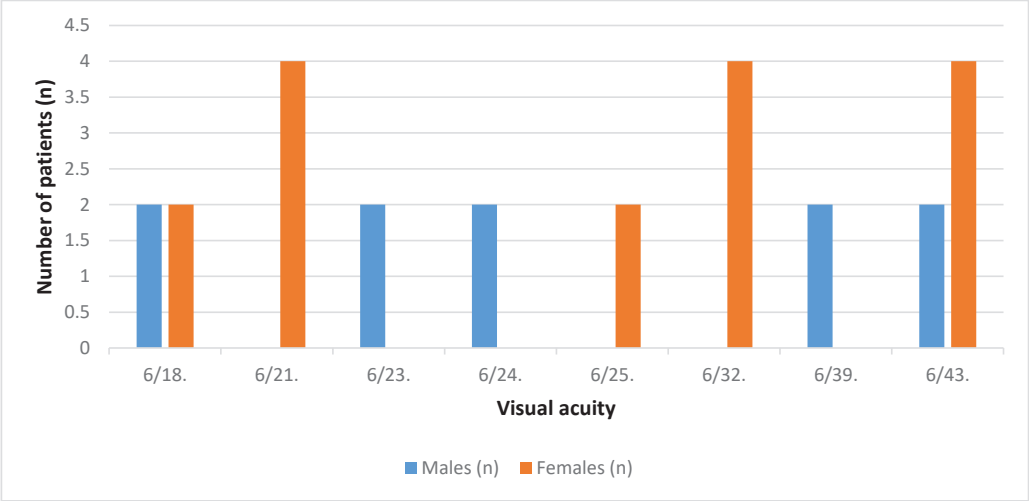

Fig. 5. The frequency of Visual Acuity in 26 eyes affected with XECD

Table 1. Age distribution of patients with Endothelial Corneal Dystrophy by gender

Age Distribution	F	%	M	%	N	%
0-10	4	4.25	7	6.73	11	5.56
11-20	10	10.64	12	11.54	22	11.11
21-30	17	18.09	17	16.35	34	17.17
31-40	14	14.89	17	16.35	31	15.66
41-50	34	36.17	29	27.88	63	31.82
51-60	12	12.77	20	19.23	32	16.16
61-70	3	3.19	2	1.92	5	2.53
Total	**94**	**100**	**104**	**100**	**198**	**100**

corneal dystrophies, 38.38 % (n=76) were of FECD from which 55.26 % (n=42) were males and 44.74 % (n=34) were females. Cases of FECD have only been observed in three age groups and the most prevalent group was 41-50 years with 57.89 % (n=44) cases among which 52.27 % (n=23) were males and 47.73 % (n=21) were females. On the other hand, the least group was 51-60 years with 5.26 % (n=4) cases and it was found to be 50 % (n=2) males and 50 % (n=2) females. In third age group 61-70 years cases were 36.84 % (n=28) which included 60.71 % (n=17) males and 39.28 % (n=11) females.

Out of 198 cases of endothelial corneal dystrophies, 25.25 % (n=50) were of CHED of which 64 % (n=32) were males and 36 % (n=18) were females. In one study PPCD appears rare and affects 1/100,000 people [3]. While in our study XECD was observed as rare.

This frequency study will help in informing the updated frequency of endothelial corneal dystrophy (ECD) in the Pakistan population. The effort done in this study will be predominantly valued in developing awareness in the patients affected with ECD which will lead to reducing the total load and pressure of the disease. ECD causes corneal transparency to deteriorate, potentially resulting in vision loss or blurriness. So, in particular, this study would raise disease awareness among patients and their families, thereby reducing the disease burden and allowing for thorough disease investigation. Appropriate clinical evaluation, initial diagnosis, genotyping, genetic awareness, and proper supervision are required for the repair of damage to

vision due to ECD.

4. CONCLUSION

Among 3000 patients, 6.6 % (n=198) cases of ECD were recognized which includes more males 52.52 % (n=104) as compared to females 47.48 % (n=94). Maximum cases of ECD 31.82 % (n=63) were observed in the 41-50 age group. In all types of ECD, FECD was observed most frequent with 38.38 % (n=76) and XECD with 6.67 % (n=13) was least frequent. The frequency of CHED and PPCD was 25.25 % (n=50) and 29.79 % (n=59) respectively. The results of recent research finalize that endothelial corneal dystrophy is a rare disease prevailing in Pakistan with a proportion of only 6.6 %. This study benefits in updating the data about the frequency of endothelial corneal dystrophy in Pakistan. Careful clinical evaluation, early diagnosis, genotyping, genetic counseling, and proper treatment are necessary for the restoration of vision loss due to ECD.

5. ACKNOWLEDGEMENTS

We sincerely thank Endothelial corneal dystrophy patients and staff of all hospitals for their assistance in the clinical evaluation and collection of data.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Adoption and Cost-benefit Analysis of Drip Irrigation for Production of High-Value Crops in Pakistan

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Abstract: Drip irrigation is regarded as one of the highly efficient methods that allow limited water resources to be properly utilized. The study has been conducted to perform the economic analysis of low and high head drip irrigation systems throughout the country based on data from 100 adopters of drip irrigation through a research & development project funded by the International Center for Agricultural Research in the Dry Areas (ICARDA-Pakistan)/United States Department of Agriculture (USDA). A field survey for the study was conducted in the year 2018 in all the provinces of Pakistan. The study revealed that technology is generally adopted by the farmers having diversified income sources and medium-sized land holdings i.e. ranging from 12.5 to 25 acres. System installation cost-shared 11 percent in low-head drip system of the annual production cost of fruits. While, it shared 29, 32, and 27 percent in case of a high-head system for grapes orchards, vegetables in tunnels, and open fields, respectively. Low-head drip irrigation is profitable for dates/ lemon orchards in Khyber Pakhtunkhwa with a benefit-cost ratio (BCR) of 1.27:1.00. It results in considerable profitability for grapes orchards with BCR of 1.73:1.00 and 1.32:100 in Punjab and Balochistan provinces, respectively. The low-head system is also beneficial for mixed fruit orchards in rain-fed Punjab with a BCR of 1.24:1.00. Similarly, for high-head systems, the benefit-cost ratio was the highest for grapes produced in rain-fed Punjab (2.62:1.00), followed by squash-gourd in irrigated Sindh (2.17:1.00) bitter-gourd in rain-fed Punjab (1.50:1.00) and okra in Sindh (1.22:1.00). However, low and high-head systems could not result in considerable returns for farmers in the case of mixed fruits in Balochistan and cucumber production in tunnels in Punjab province during the study year.

Keywords: Adoption, Benefit-cost ratios, Drip Irrigation, Fruit, High-head, Low-head, Pakistan, Vegetable

1. INTRODUCTION

Optimizing the use of water is critically important for the socioeconomic uplifting of people and the development of any country. In this perspective, increasing water scarcity and efficient use of existing water resources is becoming a global challenge [1]. As, irrigation is the artificial application of water to crops through appropriate methods such as surface (basin, border, furrow), or pressurized (sprinkler, bubbler, drip) irrigation systems [2]. While, the irrigation system of Pakistan comprises of three major reservoirs, 16 barrages, two headworks and syphons each, 12 link canals, 44 canal commands, and more than 140,000 watercourses. Although Pakistan possesses one of the largest contiguous gravity flow irrigation networks, but it is confronted with many issues such as low irrigation efficiency and water productivity, under-designed capacity, old infrastructure requiring extensive maintenance, water scarcity, inequity, etc. [1]. Basin or flood irrigation has the lowest irrigation efficiency (40-50 %), while overhead methods like sprinkler and drip/trickle are efficient in irrigation application by 60-65 % and up to 90 %, respectively [2].

It is believed that an increase in water requirement due to the rising population and

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increasing demand for industrial usage may limit the increase in agricultural productivity. One of the options for future water needs is to use available water resources more efficiently and effectively for the production of staple crops in general and high-value crops in particular [3]. Alternatively, there should be rationing on payment of the price for water management. We need to find out the solutions without hurting socio-political sensitivities. That is possible with superior strategic management through the use of appropriate technologies and awareness amongst citizens in general and farmers in particular [4]. High-efficiency irrigation system (HEIS) is an option to meet future water needs, as it results in the efficient and effective use of precious water resources, which are being overexploited. The systems are adopted in Kenya and neighboring countries to combat periodic droughts and alleviate food security for thousands of people [5]. Similarly, large-scale adoption of HIES alongside low-cost greenhouse farming technology by smallholder farms in rain-fed countries is reported [6]. HEIS such as sprinkler and drip have several advantages over surface irrigation, such as; high application efficiency & productivity, better quality production, low energy requirements, reduced soil salinity hazard & weed growth, fewer disease problem, and can be highly automated [2]. Similarly, the overview of the current water availability and irrigation system in the country highlighted drip irrigation technology as an alternate solution for sustainable irrigated agriculture [7]. Similarly, it is reported that the technology produces more crop yield by one-fourth with more than half of water use in rain-fed areas of Sindh [8]. Likewise, it is stated that the adoption of water-efficient technologies and diversifications to higher-value crops help accelerate agricultural water productivity [9].

HEIS allows water near the plant roots either onto the soil surface or beneath the soil surface directly to the root zone area. Thus, the use of HEIS also minimizes soil erosion. Application of irrigation water as well as agrochemicals according to crop requirements is possible through the system. HEIS can irrigate irregular-shaped fields without proper land leveling, as is necessary in the case of basin, border, and furrow irrigation methods. The drip irrigation allows safe use of recycled water; maintains moisture within the root zone near the field capacity. Even, the soil type plays a less important role in the frequency of irrigation application in this method. The other advantages of drip irrigation include a highly uniform distribution of water i.e. controlled by the output of each nozzle. The water system is regulated through valves and drippers hence labor cost is minimum [10].

Through HEIS, water which lies beneath the earth's surface or not at the proper height from the field surface is provided through suitable pressure generated by electricity or diesel pumps. This passes through main, sub-main, lateral lines and emitters in a sequence and reaches in drops near plant roots. Emitters are placed at a recommended distance for different vegetables. Similarly, the use of lateral pipes with holes at suitable gaps is also becoming popular. As fruit and vegetables are usually planted in rows, thus they can be easily irrigated through this system. This system can also be used with mulching in open fields or tunnels [11]. Through this system, farmers can use available irrigation water in a better way to obtain higher productivity and profitability from tunnel farming. It is quite simple technology, which is most efficient and easy to use, increases crop yield up to 25 percent; applicable for vegetables, orchards, and cotton crops; and is equally suitable for kitchen gardening [12]. Similarly, drip irrigation provides the salinity control for the profitable irrigation of vegetables [13]. These systems, however, also have some constraints such as high initial cost, maintenance requirements, restricted plant root development, salt accumulation near plants (along the edges of the wetted zone), application of insoluble or slightly soluble fertilizers such as Super Phosphate or Sulphate of Potash is not readily possible [2]. While the most severe problem is the clogging of emitters by particulate and biological materials and this can cause poor application uniformity [14]. However, flushing the system after each cropping season can solve this problem [2].

Since 1985, the Government of Pakistan has initiated several projects to promote sprinkler and drip irrigation systems in the country. However, most of these projects could not achieve the intended objectives due to; high capital cost, non-availability of material, no backup support, complicated/ over-designed systems, small landholdings, and lack of knowledge about irrigation scheduling (when to apply and how much water to apply to crops),

farmers' misconception about the system, and a flat rate of electricity in case of Balochistan. Therefore, for the success of these systems, the selection of the right area, right crop, right farmer, right material, and right design are very important. Moreover, the design of the system should be simple, so that common farmers can easily maintain and operate it. Potential areas for these systems include the Pothwar plateau, desert, semi-desert areas, uplands of Balochistan, riverine belts, greenhouses, and tunnels [2].

Low-head drip irrigation systems are generally permanent and have low labour and energy requirements [14]. Low head drip irrigation is a viable technology for rain-fed areas, as its use helps farmers to efficiently use limited water resources in these semi-arid and arid areas [15]. Low-head drip irrigation for high-value orchards is being promoted by the Pakistan Council of Research in Water Resources (PCRWR) Head Quarters, Islamabad, and its Regional Office, Peshawar and Regional Office, Quetta, also called Water Resources Research Centre (WRRC) in rain-fed areas of Punjab (Attock, Rawalpindi and Chakwal districts), Khyber Pakhtunkhwa (Dera Ismail Khan district) and Balochistan (Quetta, Mastung and Killah Abdullah districts), respectively. High-head drip systems have high energy requirements as water storage is generally more elevated than low-head systems thus water supply is also more pressurized. Soil and Water Conservation Research Institute (SAWCRI), Chakwal and Climate, Energy and Water Research Institute (CEWRI), PARC-National Agricultural Research Centre, Islamabad promotes high-head drip irrigation for orchards in rain-fed areas of Punjab (Chakwal district). Similarly, high-head drip irrigation systems for vegetables are being promoted by the Institute of Water Resources Engineering and Management (IWREM), Mehran University of Engineering and Technology (MUET), Jamshoro with the support of a local support organization, named Rural Development Foundation (RDF) in the irrigated area of Sindh province (Jamshoro district).

Keeping in view the water scarcity situation in the country, the International Center for Agricultural Research in the Dry Areas (ICARDA) and the United States Department of Agriculture (USDA) launched the project 'Diffusion and adoption through partnership and action of the best watershed rehabilitation and irrigation practices and technologies to help rural farmers' to promote adoption of HEIS and other water saving/ soil moisture conservation technologies. ICARDA, Pakistan office through local technical partner institutes viz. PCRWR, PARC, SAWCRI and MUET, disseminated the pertinent knowledge and demonstrated drip irrigation systems throughout the country in the first phase of the project (2014-16). In the second phase (2017-18) farmers were convinced to adopt HEIS by developing their linkages with materials /service providers, and technical experts, and also by providing subsidies at a rate of 60:40, i.e. 60 % of the cost was borne by the ICARDA/USDA in Dera Ismail Khan district, and Jamshoro district of Sindh, at a rate of 70:30, i.e. 60 % of the cost was borne by the ICARDA/ USDA and 10% was borne by RDF. Subsidy for the adoption was provided to the farmers keeping in view their resource poverty at both sites, and also due to the tough terrain of the area in the Dera Ismail Khan district. Consequently, a considerable number of adoptions were reported from different institutes including PCRWR, CEWRI-PARC, SAWCRI, etc. As very little has been published about the cost-benefit analysis and adoption of the technology in the country. Thus, the article is an effort to document the demographic characteristics and livelihood sources of the adopters; their knowledge level and adoption of the technology, area allocation to drip systems, and cost-benefit analysis of the technology across provinces. The findings of the research work are helpful for public/ private sector stakeholders to fine-tune their plans/ policies to up-scale adoption of the technology in the country, and for farmers to adjust their investment priorities in drip irrigation.

2. MATERIAL AND METHODS

The study is based on a field survey, data collected from 100 adopter farmers (about 17 % of a total, 597 adoptions) that were randomly interviewed out of the lists of the adopters provided by the technical institutions promoting the technology in the country. The sample for the study has been drawn based on number of total adoptions, spread of adoptions and availability of human, financial and logistic resource by PARC Satellite institutes, Social Sciences Research Institute in Islamabad, and countrywide technical partners. The sample for

the study includes 50 farmers each of low-head and high head drip irrigation systems. Field surveys for the study were conducted from January 2018 to May 2018 through a set of well-structured and pre-tested questionnaires that were prepared in consultation with technical experts and socio-economists from regional offices of ICARDA in Pakistan and Jordan, as well as technical experts from national and provincial agricultural research systems. Detailed questionnaires were developed for the study to obtain information about farmers' knowledge about the technology, their practical experiences, suitability of the technology in the study areas, economic gains of adoption, and adoption potential covering fellow farmers' interest in its adoption and possible constraints in up-scaling of the technology.

Distribution of the sample respondents across provinces along with details about the type of drip irrigation system, commodities (fruits and vegetables), etc. are presented in Table 1. In rain-fed areas of the Punjab and Balochistan provinces, low-head systems are used for high-value orchards of grapes and other fruits. While, in rain-fed areas of Khyber Pakhtunkhwa province, the system is used for date and lemon orchards that are generally planted in a mix. While high-head systems are used for grapes, tunnel farming of cucumber and bitter-gourd in rain-fed Punjab, and for production of okra and squash gourd in open fields in irrigated areas of Sindh. Details about sample farmers across provinces along with study areas and orchard types are presented in Table 1.

Low-head drip irrigation technology has been promoted through financial support/subsidy in the Dera Ismail Khan district of Khyber Pakhtunkhwa. Thus, sample farmers are inquired in detail about the establishment and maintenance costs of high-value orchards. Date plants generally take three to five years to bear a marketable level of the produce, thus production, marketing, and income data were substantiated by the information about already established fruit-bearing orchards in the study area, that were mature enough and in the full fruit-bearing stage. In this region and other parts of the country, along with thorough information that was collected through structured questionnaires, detailed information about a few aspects of adoption prospects of the technology has also been obtained through discussion with the respondents. The data has been analyzed for descriptive statistics and cost-benefit analysis through SPSS-22 and MS-Excel, respectively.

3. RESULTS AND DISCUSSION

Adopter farmers of HEIS were in the middle age group, with a mean age of 42.2 years and formal education of 9.5 years (Table 2). They were involved in crop and fruit farming for a long period of time, with a mean farming experience of 18.0 years. Mean family size of the sample farming households was 8.9. Mean operational holding of the farmers was 14.8 acres. This means that generally the technology is adopted by medium-size farms, having landholding of greater than 12.5 acres. Thus, small farmers could not adopt it, while

Table 1. Distribution of the sample across provinces with type of drip irrigation system

Type of System	Province	District (s)	Study Area (Villages)	Orchard Type	Sample Size
Low-head system for high value orchards (Rain-fed)	Khyber Pakhtunkhwa	Dera Ismail Khan	Wanda Feroz & Panyala	Dates/ Lemon	10
	Punjab	Attock	Narthopa, Salar, Pandak & Malikmal	Grapes & Mixed Fruit	20
		Rawalpindi	Wah/ Wah Gardens Town		
		Chakwal	Dhok Darbi		
	Balochistan	Quetta	Bazee, Merabzae, Hassanabad & Sajawalabad	Grapes & Mixed Fruit	20
Sub-total					50
High-head system for orchards and vegetables (Rain-fed)	Punjab	Chakwal	Thati Gujran & Fatehjang	Grapes,	30
				Cucumber & Bitter-gourd	10
High-head for orchards and vegetables (Irrigated)	Sindh	Jamshoro	Bhit Kacchi & Shirabad	Okra & Squash-gourd	10
Sub-total					50
Total					100

large farmers usually rely on major crops for their income, and do not prefer to adopt capital-intensive and technical skills requiring technologies. The average area under drip irrigation at the sample farms was 4.2 acres, 67.7 % of the irrigated area at these farms. Drip irrigated area as a percent of the total irrigated area was minimum in irrigated Sindh (19.2 %). While in rain-fed areas of Khyber Pakhtunkhwa and Balochistan irrigated area was solely limited to drip systems. As the adoption of technology is mainly limited to rain-fed areas, thus due to the limitation of cropland farming families keep livestock in very limited numbers, with a mean number of animals at sample farms of three. Technology type and province-wise details are presented in Table 2.

Sample farming households have diversified sources of livelihood. Details about income sources along with contribution to the total income of the sample farmers by regions are given in Table 3. Crops' share in the income of the sample farmers who have had installed drip irrigation systems at their farms was 55 % followed by job and remittances (19.4 %), small enterprises and trade (14.2 %), livestock (9.2 %), and farm labour and services (2.2 %). The share of the crop income of farmers was the highest in Balochistan (99 %), followed by in irrigated Sindh (55.7 %) and rain-fed areas of Punjab (43.3 %) and Khyber Pakhtunkhwa (39.0 %). Livestock shared the highest in irrigated Sindh (27.7 %) followed by rain-fed areas of Punjab (9.9 %) and other provinces. Farm labour and services as well as small enterprises and trade shared the highest income of the farmers in rain-fed areas of Khyber Pakhtunkhwa (11.0 %). While job and remittances contributed to the maximum income of farmers in rain-fed areas of Punjab (42.3 %).

ICARDA-USDA funded initiative for the promotion of drip irrigation created awareness in far-flung areas of the country. All the sample farmers in Balochistan, Khyber Pakhtunkhwa, and Sindh provinces reported that technical persons working on the project made them aware of the benefits of the adoption of the technology (Table 4). In Punjab province, On-Farm Water Management (OFWM) department has created awareness in the farming community about the technology on a large scale. About half of the sample adopter farmers (49 %) reported obtaining proper training in drip system installation. Farmers' adoption experiences vary greatly across provinces, with the lowest of one year in Sindh to the highest of 4-5 years in Punjab. Availability of technical support for installation as well as for repair and maintenance of the systems was excellent in Punjab and Sindh provinces, with 100 % response by the farmers about access to these services. While, one-third and half of the sample farmers in Khyber Pakhtunkhwa and Balochistan provinces reported access to technical support services about the technology, respectively. The interest level of the fellow farmers of the adopters in the technology was medium to high (Table 4).

In the case of a low-head drip system in Khyber Pakhtunkhwa, as far as access to market and enabling institution is concerned, materials and equipment for drip irrigation are not available in local markets and farmers are to purchase these mostly from Peshawar. However, the mean distance of local input markets from sample farms was five kilometers. One-third of the farmers (33%) reported that technical support for the adoption of the technology is readily available to them, and they have access to at least two Agricultural Service Providers (ASPs) or technicians in their vicinity. All these farmers declared that the availability of services for installation, repair, and maintenance of the drip irrigation systems are sufficient to fulfill their requirements.

Farms of the sample adopters in the case of low-head drip irrigation in Punjab were located far away from local input as well as output markets, with an average distance of more or less 40 km for each. While the mean distance of the farms from non-local input/output markets was 119 km. The main constraints in the adoption of the technology on large scale in the study area are the high cost of installation of the system, non-availability of material in local markets, and lack of services of technical people /ASPs, as reported by 33 percent of the sample farmers each. In the case of a drip irrigation system for fruit plants in the province, sample respondents reported that at least one of their fellow farmers adopted the technology by looking after its success on their farms. They reported that they have access to two to three ASPs each; however, as most of these are serving the drip system installation companies, they are only occasionally available to them.

Table 2. Demographic characteristics of the farmers

Characteristics		Low-head Systems		High-head Systems		Total	
		Khyber Pakhtunkhwa (n=10)	**Punjab** (n=20)	**Balochistan** (n=20)	**Punjab** (n=40)	**Sindh** (n=10)	**Pakistan** (n=100)
Age (year)		46.3 (15.9)	37.0 (8.2)	42.8 (7.6)	40.0 (11.9)	44.8 (11.0)	42.2 (10.9)
Education (year)		8.0 (6.9)	8.7 (5.2)	9.7 (4.5)	11.0 (5.1)	10.0 (2.8)	9.5 (4.9)
Farming experience (year)		18.3 (14.4)	11.8 (7.1)	16.3 (15.0)	17.7 (14.2)	26.0 (9.6)	18.0 (12.1)
Family size (number)		7.0 (2.8)	7.2 (3.8)	13.2 (4.2)	8.2 (3.9)	8.8 (2.4)	8.9 (3.4)
Operational land holding (acre)	Total	11.4 (9.9)	11.6 (7.9)	10.4 (4.0)	35.3 (32.6)	5.2 (2.0)	14.8 (11.9)
	Rain-fed	10.1 (9.6)	4.2 (6.7)	3.0 (4.7)	25.9 (27.7)	0.0 (0.0)	8.6 (10.4)
	Irrigated	1.2 (0.7)	7.4 (6.9)	7.4 (2.0)	9.4 (5.7)	5.2 (2.0)	6.2 (3.2)
Drip irrigation area (hectare)		1.2 (0.7)	6.4 (1.7)	7.4 (6.9)	3.0 (2.0)	1.0 (0.7)	4.2 (3.0)
Drip area as % of op. holding		100.0	86.5	100.0	31.9	19.2	67.7
Livestock holding (number)		4.0 (2.0)	2.8 (3.0)	0.5 (0.8)	3.5 (4.6)	5.0 (4.3)	3.2 (2.3)

Source: Field Survey 2018, Pakistan Note: Figures in parenthesis are standard deviations

Table 3. Sources of Income (percent)

Sources	**Low-head Systems**		**High-head Systems**		**Total**	
	Khyber Pakhtunkhwa (n=10)	**Punjab** (n=20)	**Balochistan** (n=20)	**Punjab** (n=40)	**Sindh** (n=10)	**Pakistan** (n=100)
Crops	39.0	38.2	99.0	43.3	55.7	55.0
Job & Remittances	13.2	42.3	0.0	34.2	7.4	19.4
Small enterprises & trade	35.7	13.3	0.0	12.6	9.2	14.2
Livestock	1.1	6.2	1.0	9.9	27.7	9.2
Farm labour & services	11.0	0.0	0.0	0.0	0.0	2.2

Source: Field Survey 2018, Pakistan

Table 4. Knowledge of the technology and its Adoption

Characteristics		**Low-head Systems**		**High-head Systems**		**Total**	
		Khyber Pakhtunkhwa (n=10)	**Punjab** (n=20)	**Balochistan** (n=20)	**Punjab** (n=40)	**Sindh** (n=10)	**Pakistan** (n=100)
Source of information (%)	Project	100	10	100	17	100	49
	OFWM	-	90	-	83	-	51
Trained (%)		33	60	33	17	100	49
Experience (years)		2.0	5.0	2.0	4.0	1.0	3.0
Technical Support (%)		33	100	50	100	100	83
Fallow farmers' interest (%)	High	0	70	0	67	80	49
	Medium	30	30	80	33	10	39
	Low	70	0	20	0	10	12

Source: Field Survey 2018, Pakistan

Drip irrigation technology is quite new for the adopter farmers in Sindh province, as they reported to get knowhow about it since the end of the year 2017 through farmer field days organized by Institute of Water Resources Engineering and Management (IWREM), MUET, Jamshoro in collaboration with a Local Support Organization (LSO) named Rural Development Fund (RDF). They declared these events very much informative and effective in dissemination of the knowledge about the technology. All the sample farmers reported attending training programs jointly organized by IWREM and RDF on drip irrigation for vegetables. All of them reported visiting demonstration sites/ fields of their fellow farmers to practically witness the working of the technology. Sample farmers reported that most of their fellow farmers (80 %) are highly interested in the adoption of the technology. However, they proposed to increase the subsidy for adoption from 70 % to 80 %. Drip irrigation is declared a future lifeline for Pakistan's irrigated agriculture [7]. However, they stated that high initial costs and lack of awareness hamper the adoption of this technology. In the case of low head drip irrigation in Balochistan, adopter farmers reported that the technology is very beneficial and most of their fellow farmers (80 %) are taking a medium level of interest in up-taking the technology in the province. Half of the adopter farmers reported access to services of one ASP each for installation and repair and maintenance of the drip systems at their farms.

Thus, the numbers of ASPs in the study area are insufficient to meet farmers' demand for services. The results are in line with [16], they reported low adoption of the technology due to lack of awareness in farmers, incapability of ASPs to serve farmers, small landholdings, the poor resource base of the farmers, and social/cultural non-acceptance. Sample farmers reported that the adoption of the technology is not being promoted by public sector development projects. However, half of them (50 %) reported that few NGOs are making efforts to promote the technology in the study area.

Area allocation to drip irrigation system by types of fruit or vegetables is given in Table 5. It ranged from the lowest of 1.0 acre for okra/ squash-gourd in Sindh province to 1.25 for cucumber/ bitter-gourd in Punjab province. Adopter farmers of high-head HEIS of Sindh province reported that they can double the area under drip irrigation for vegetables if the subsidy program by the local LSO continues to support them in adoption. In the case of fruit crops, areas under HEIS ranged from 1.14 acres in Khyber Pakhtunkhwa province to a maximum of 8.9 acres in Punjab.

Cost-benefit analysis of low head drip irrigation by fruit types and provinces is given in Table 6. The analysis has been summed up at the country level in Figure 1. It is found that water pump installation and construction of raised tank/ storage reservoir, and land cost/rent are the main cost item in the production of fruits through the adoption of HEIS (each shares 22 % of the total annual cost of production each). Irrigation cost is the second major cost item and shares 18 percent of the total cost. Drip material and HEIS installation cost is the third main cost item with a share in the total cost of production of 11 percent. Water pump installation cost varies greatly across provinces, as it depends on bore depth and topographic/ geographic conditions. It ranged from Rs. 185,000 to Rs. 1,205,000 (Table 6). It was the lowest in Punjab province followed by Khyber Pakhtunkhwa province, with the highest in Balochistan province. Land preparation, planting material and planting cost-shared eight percent of the total cost of production. Fixed cost for fruit production through a low-head drip system was the lowest in Punjab province for mixed fruit orchards (Rs. 18,490) and was the highest in Balochistan province for grape orchards (Rs. 68,592).

The total cost of production/acre was the lowest for mixed fruit orchards in rain-fed Punjab province (Rs.74,460) and was the highest for dates and lemon orchards in Khyber Pakhtunkhwa province (Rs. 154,799). Gross revenues ranged from Rs. 69,590, for mixed fruit orchards in Balochistan to Rs. 254,373 for grapes orchards in Punjab province. Similarly, net revenue was the highest for grapes orchards in Punjab province (Rs. 106,696), with the lowest for grapes orchards in Balochistan (Rs. 41,330). The benefit-cost ratio was the highest for grapes orchards in Punjab i.e. 1.73: 1.00, followed by grapes orchards in Balochistan, date and lemon orchards in Khyber Pakhtunkhwa. While farmers having mixed fruit orchards with low-head drip installation in Balochistan faced a loss of Rs. 20,054 per acre due to low harvest prices.

Cost-benefit analyses of high-head drip irrigation systems used for the production of grapes in rain-fed Punjab and selected vegetables produced through tunnel farming in rain-fed Punjab and open fields in Sindh are given in Table 7. Share of cost items for high-head drip irrigation systems used in grapes orchards in rain-fed Punjab are presented in Figure 2. Drip material and installation and land preparation, planting material, and planting

Table 5. Area allocation to drip irrigation system by orchard/ vegetable types

Type of System	Province	Orchard/ Vegetable Type	Mean Area (acre)		
			Mini-mum	Maxi-mum	Mean
Low-head for high value orchards (Rain-fed)	Khyber Pakhtunkhwa (n=10)	Dates & Lemon	0.50	1.50	1.14
	Punjab (n=20)	Grapes	1.00	9.00	4.69
		Mixed Fruit	5.25	12.50	8.90
	Balochistan (n=20)	Grapes	1.25	19.0	7.17
		Mixed Fruit	1.00	4.00	2.22
High-head for orchards and vegetables (Rain-fed)	Punjab (n=40)	Grapes (30)	1.00	5.00	3.00
		Cucumber / Bitter-Gourd (10)	1.00	2.00	1.25
High-head for vegetables (Irrigated)	Sindh (n=10)	Okra / Squash-Gourd	1.00	1.00	1.00

are major cost items, shared 29 % each in the total annual cost of production farmyard manure & fertilizers and irrigation are other main cost items with shares in the total cost of production of 15 and 12 % respectively. Share of cost items for a high-head drip irrigation system for vegetable production in tunnels in rain-fed Punjab are given in Figure 3. Drip material and installation, and labor are major cost items and shared 32 and 15 % in the cost of production of vegetables (cucumber and bitter-gourd). Plastic tunnel cost is the third major cost item of the vegetables with a share of 11 percent. Similarly, marketing costs constitute 9 % of the total cost of production in vegetable tunnel farming. Share of cost items for a high-head drip irrigation system for vegetable production in open fields in irrigated Sindh are presented in Figure 4. Land cost/ rent, and drip material & installation costs are major cost items, shared 30 % and 27 % in the total cost of production, respectively. Irrigation, and farmyard manure & fertilizers costs shared 14 % and 8 % of the total cost, respectively. While others cost types can be considered as minor items with a share in the total cost of production of seven percent or less. In a high-head drip irrigation system, the total cost of production per acre was the lowest for okra production in open fields in irrigated Sindh (Rs. 73,243), with the highest for cucumber production in tunnels in rain-fed Punjab (Rs. 165,453). Gross revenues per acre ranged from Rs. 89,100 for okra production in Sindh to Rs. 246,160 for bitter-gourd produced in tunnels in rain-fed Punjab.

While, net revenue was the highest for grapes in rain-fed Punjab (Rs. 148,563 per acre), followed by squash-gourd produced in the open field in irrigated Sindh, and bitter-gourd in rain-fed Punjab. Farmers producing cucumber in tunnels had to bear a loss of Rs. 8,753 per acre due to the high cost of

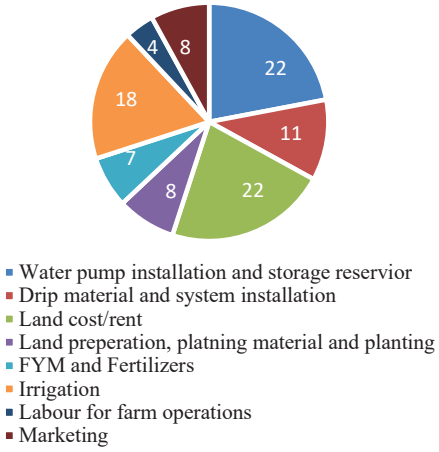

Fig. 1. Cost shares for fruit low-head drip irrigation (%)

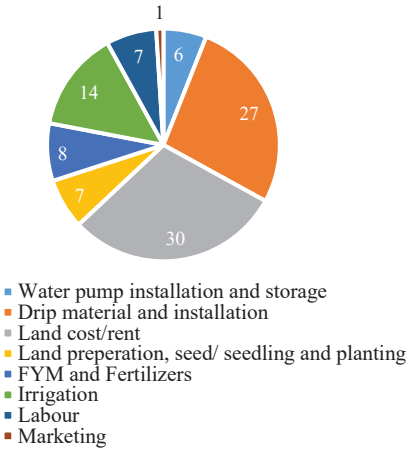

Fig. 2. Cost shares for high-head grape orchards (%)

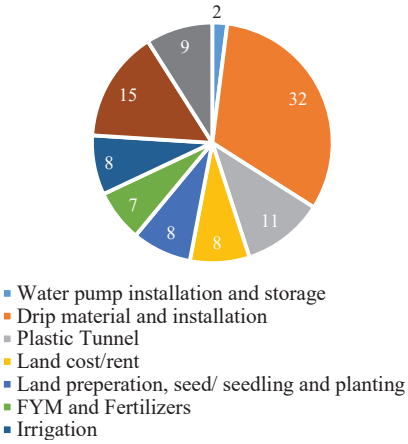

Fig. 3. Cost shares for vegetables tunnels in rain-fed Punjab (%)

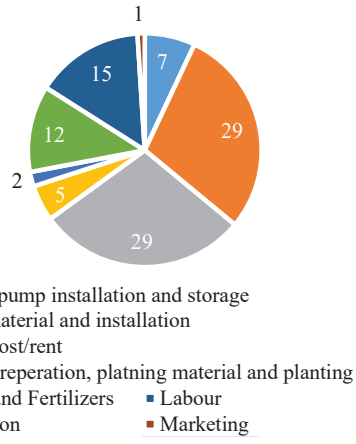

Fig. 4. Cost shares for high-head irrigation for vegetables in open fields in Sindh (%)

Table 6. Cost-benefit analysis of low-head drip Irrigation (Rs. per acre)

Costs Items with specification	Units	Quantity Range	Cost per farm/ Price Range	Life Range (year)	Value per annum				
					Khyber Pakhtun-khwaDates & Lemon	Punjab		Balochistan	
						Grapes	Mixed Fruit	Grapes	Mixed Fruit
Fixed Cost					37248	38118	18490	68592	52338
Raised tank/ reservoir (Concrete)	No.	1	65300-350000	20-25	2605	2500	1966	1395	4505
Water Pump (Peter Engine / Sub-immersive)	Rs..	-	185000-1205000	20-30	23754	8000	4757	40418	39351
Installation									
Conveying Pipes (PVC 2")	Foot	58-2285	95-170	20	432	530	226	1705	1687
Sub main (PVC 1.5"/2.0")	Foot	119-641	34-71	10-20	1238	1817	4106	4720	941
Drip lines (Other 0.75")	Meter	200-1200	7-70	10	3684	170	840	658	1058
Drippers	No.	97-633	9-20	4-10	658	813	593	194	483
Filter	No.	1	8000-20667	10	-	2067	800	-	-
Valves	No.	1-2	1700-28333	10-20	283	557	1000	1417	1600
Supporting pillars	No.	200-250	300	7	-	8526	-	10714	-
Installation cost	Rs.	-	26315	10	3000	3305	3652	2521	2278
Suckers (Dates)	No.	54	650	25	1414	-	-	-	-
Plants (Lemon)	No.	18	250	25	180	-	-	-	-
Plants (Grapes)	No.	420-485	100	10-20	-	9833	-	4850	-
Plants (Mixed Fruits)		87-110	100	20	-	-	550	-	435
Variable Cost					117551	109259	55970	58718	37306
Land Cost/ Rent	acre	1	10927	1	23684	26358	26358	27000	27000
Land Preparation	Rs.	1	8772	1	8772	6631	3652	2200	1051
Labour (Planting)	M. days	1.8-4.8	400-700	1	1930	2300	2760	2668	1022
FYM (Basal dose)	Trolley/ Trucks	1.1-3.6	2250-4350	20-25	313	405	2520	-	-
FYM (Top dressing)	Trolley/ Trucks	2.6	4350	1	11310	-	-	-	-
Gypsum	Bags	1.8	200	1	-	360	-	-	-
Fertilizer 1 (Urea)	Bags	0.78-2.42	1300-1725	1	1488	2769	1287	4175	
Fertilizer 2 (DAP)	Bags	0.85-2.42	2700-3400	1	2856	2720	2700	7260	
Fertilizer 3 (SP)	Bags	0.73	3000	1	-	-	2190	-	-
Fuel (Diesel)/ Elect. Bill (Rs.)	Liter	101-475	105	1	41475	49875	10605	1620	1623
Labour (Interculture, pruning & pollination & fruit picking	M. days	2.76-26.00	400-600	1	10386	3875	2550	2526	2316
Marketing cost	Rs.	1	15337	1	15337	13966	1348	11269	4294
Total Cost					**154799**	**147377**	**74460**	**127310**	**89644**
Production and Revenue									
Dates					159880	-	-	-	-
Consumed	Rs./kg	35	200	1	7000	-	-	-	-
Output-sold	Rs./kg	1274	120	1	152880	-	-	-	-
Lemon					36667	-	-	-	-
Consumed	Rs./kg	18	100	1	1800	-	-	-	-
Output-sold	Rs./kg	498	70	1	34867	-	-	-	-
Grapes					-	254373	-	168640	-
Consumed	Rs./kg	63-79	70-127	1	-	10033	-	4410	-
Output-sold	Rs./kg	2572-2986	55-95	1	-	244340	-	164230	-
Mixed Fruits					-	-	92425	-	69590
Consumed	Rs./kg	135-166	110-135	1	-	-	18225	-	18260
Output-sold	Rs./kg	742-870	59-100	1	-	-	74200	-	51330
Gross Revenue					**196547**	**254373**	**92425**	**168640**	**69590**
Net Revenue					**41748**	**106996**	**17965**	**41330**	**-20054**
Benefit-cost ratio (Gross Revenue/ Total Cost)					**1.27**	**1.73**	**1.24**	**1.32**	**0.78**

Source: Field Survey 2018, Pakistan

Note: Marketing cost includes Load/ unloading, transportation and commission)

Table 7. Cost-benefit analysis of high-head drip irrigation (Rs. per acre)

Costs Items with specification	Units	Quantity Range	Cost per farm or Price Range	Life Range (yrs.)	Value per annum				
					Punjab Grapes	Punjab Cucum-ber	Punjab Bitter-gourd	Sindh Okra	Sindh Squash-gourd
Fixed cost					34392	81059	81059	20370	35106
Storage reservoir (Concrete)	No.	1	100000-120000	20	1667	-	-	-	-
Water Pump (Peter Engine / Sub-immersive) Installation	No.	1	105000-281400	5-20	5000	4167	4167	5400	5400
Conveying Pipes (PVC 2"/ Galvanized steel 3"/Copper2")	Foot	220-371	70-550	10-20	3850	991	991	2000	20387
Sub main (PVC 0.5"/1.0"/2.25")	Foot	41-384	42-70	10-20	258	1792	1792	770	319
Drip lines (PVC 0.25"/0.5")	Meter	800-2943	25-70	10	3360	7358	7358	8400	4000
Drippers	No.	1067-3885	30-40	5-10	6097	23210	23210	1200	1333
Filter	No.	1	45000-50000	10	4500	20235	20235	-	-
Valves/Ventury	No.	1-4	6250-15000	10	625	948	948	600	667
Supporting pillars	No.	133	400	7	5700	-	-	-	-
Plastic tunnels cost	Rs.	1	20334	1	-	20334	20334	-	-
Installation cost	Rs.	1	15000-25000	10	2000	2024	2024	3000	3000
Plants (Grapes)	No.	267	100	20	1335	-	-	-	-
Variable Cost					57487	84394	82768	52873	56831
Seed/ Seedling cost	Rs.	1	2100-8130	1	-	8130	6504	2750	2100
Land Cost/ Rent	Rs.	1	27000	0.5-1	27000	13500	13500	24423	24423
Land Preparation	Rs.	1	3000	1	3000	4065	4065	2200	2200
Labour (Planting)	M. days	5-14	200-600	20	420	3252	3252	1000	1000
FYM (Basal dose)	Trolley	0.4	7000	1	-	2800	2800	-	-
Fertilizer 1 (Urea)	Bag	1-3	1400-1650	1	1650	1650	1650	2800	4200
Fertilizer 2 (DAP)	Bag	0.3-0.4	2800-3300	1	-	1320	1320	-	924
Fertilizer 3 (SP)	Bag	2.5	2850	1	-	7125	7125	-	-
Fertilizer 4 (Nitrophos)	Bag	0.66-1.5	2400	1	-	-	-	3600	1584
Fuel (Diesel)/ Elect. Bill (Rs.)	Liter	100-125	105	1	10815	13125	13125	10500	12600
Labor (Interculture, pruning & pollination)	M. days	2.67-25	550- 600	1	1602	3339	3339	3000	5000
Labor (Fruit picking)	M. days	8-20	550-600	1	12000	9900	9900	1600	1800
Marketing cost	Rs.	1	1000-16188	1	1000	16188	16188	1000	1000
Total Cost					**91879**	**165453**	**163827**	**73243**	**91937**
Production and Revenue									
Grapes					240460	-	-	-	-
Consumed	Kg.	100	123	1	12300	-	-	-	-
Output-sold	Kg.	2480	92	1	228160	-	-	-	-
Cucumber					-	156700	-		
Consumed	Kg.	320	40	1	-	12800	-	-	-
Output-sold	Kg.	5756	25	1	-	143900	-	-	-
Bitter-gourd					-	-	246160		
Consumed	Kg.	352	60	1	-	-	21120	-	-
Output-sold	Kg.	5626	40	1	-	-	225040	-	-
Okra					-	-	-	89100	-
Consumed	Kg.	40	30	1	-	-	-	1200	-
Output-sold	Kg.	5860	15	1	-	-	-	87900	-
Squash-gourd					-	-	-	-	199600
Consumed	Kg.	40	40	1	-	-	-	-	1600
Output-sold	Kg.	9900	20	1	-	-	-	-	198000
Gross Revenue					**240460**	**156700**	**246160**	**89100**	**199600**
Net Revenue					**148563**	**-8753**	**82333**	**15857**	**107663**
Benefit-cost ratio (Gross Revenue/ Total Cost)					**2.62**	**0.95**	**1.50**	**1.22**	**2.17**

Source: Field Survey 2018, Pakistan Note: Marketing cost includes Load/ unloading, transportation and commission)

production and low output prices. The benefit-cost ratio was the highest for grapes produced in rain-fed Punjab (2.62:1.00), followed by squash-gourd in Sindh (2.17:1.00) bitter-gourd in rain-fed Punjab (1.50:1.00) and okra in Sindh (1.22:1.00). The results are in line with [17] as they declared that drip irrigation has a greater scope for the production of seasonal as well as off-season vegetables, especially in water-scarce areas of Pakistan. Similarly, drip irrigation was recommended for areas with marginal to poor quality groundwater to obtain high crop production and water use efficiency [18].

4. CONCLUSION AND RECOMMENDATIONS

Drip irrigation is a promising water-saving technology. It is being admired by the farmers predominantly in rain-fed areas of the country. However, the high initial cost is reported as the main constraint in its adoption. Moreover, the limited availability of materials and services required for the installation of the drip irrigation systems, and to keep these systems in an operational state also hamper wide-scale adoption of the technology. Farmers in far-flung areas of Khyber Pakhtunkhwa, Sindh, and Balochistan with tough terrain are resource-poor, and cannot afford to adopt the technology on their own. On-Farm Water Management is promoting the technology by providing technical support and subsidy to farmers in Punjab province. However, farmers were concerned about the quality of the materials, and the estimation of installation cost by the companies contracted by the department for the installation of the systems. Farmers still consider it a little complex technology to adopt, operate and maintain. It is required to build their confidence in the usefulness of the technology, awareness events for potential adopter farmers, and training sessions for local technical staff and plumbers should be organized. This will create demand for materials and services, as well as help, improve the supply of materials, and repair & maintenance services at the local level. Farmers' capacity may also be built for minor trouble shootings in drip irrigation systems. In irrigated areas of the country, farmers are used to flooding irrigation of field crops. Thus, they still consider that drip irrigation systems do not fulfill the water requirement of the crops and result in weak/ stunted root growth hence low productivity. Wide-scale demonstration of the technology, as well as research, is required to convince farmers that drip irrigation has a clear advantage over traditional flood irrigation systems. Farmers should also be persuaded that they can reduce the installation, and operational cost of HIES, and ultimately crop production costs either through proper utilization of high-efficiency irrigation systems or area expansion under these systems at their farms. Similarly, policy formulators and development institutions/ agencies should carefully consider crop zoning in devising their plans for the promotion of technology in the country.

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6. CONFLICT OF INTEREST

There is no conflict of interest among the authors of the article.

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Research Article

Impact of *Stevia rebaudiana* Culturing in Liquid Medium: Elevation of Yield and Biomass, Mitigation of Steviol Glycosides

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Abstract: An efficient micropropagation system is developed by culturing nodal segments containing the axillary shoot buds on liquid Murashige and Skoog (MS) medium. Direct shoot and root formation are highly effective in both solid and liquid MS media without any plant growth regulators (PGRs). Interestingly, a significant difference in yield is obtained between solid and liquid cultures. It is revealed that a relatively higher amount of plant biomass is obtained after culturing for 4 weeks in a liquid MS medium. However, the shoots produced on solid MS medium produce a remarkable decline in all physiological parameters. On contrary, the bioactive steviol glycosides (rebaudioside-A and stevioside) content is higher in shoots grown in solid MS on a comparative basis, which could be compensated by higher yield. After hardening off, all the regenerants are effectively grown in the field with a negligible loss (<1 %), and steviol glycosides spectra is again obtained by conducting high-performance liquid chromatography (HPLC) analysis after 10 weeks of the plantation. This method has great potential to be applied on large scale in bioreactors.

Keywords: *Stevia rebaudiana*, Micropropagation, Liquid Culturing, Steviol Glycosides

1. INTRODUCTION

Stevia rebaudiana, belonging to the family Asteraceae, has common names viz. sweet leaf or candy leaf [1]. It is a perennial, bushy shrub native to Northeast Paraguay [2]. *Stevia* is of great commercial importance all around the globe because of the presence of steviol glycosides (SGs) in its leaves that play a vital role in reducing high blood pressure, diabetes (Type II), obesity, and issues related to dentistry [3, 4]. This naturally occurring zero-calorie sweetening compound is involved in the regulation of glucose and insulin in the blood which is crucial in maintaining a balanced diet for the well-being of humans [5]. The major steviol glycosides (SGs) responsible for approximately 300 times more sweetness than sucrose include rebaudioside A (Reb-A), stevioside (ST), and rebaudioside C (Reb-C) [6]. SGs are the potential bioactive compounds that have been approved in various countries because of their tremendous activity against microbes, cancer, inflammation, and diabetes [4, 7].

S. rebaudiana is very beneficial to plants both economically and commercially. Conventional methods for *Stevia* propagation involving vegetative propagation through stem cuttings are very slow. Another big hurdle is that *Stevia* seeds are very tiny and poor in germination [8]. The efficacious production of *Stevia* is limited by these factors, therefore, the adoption of modern techniques of biotechnology is the only solution. Tissue culture is one such biotechnological approach having the ability to mass propagate *Stevia* in a minimum period. In this process, true-to-type progeny is produced from a single mother plant [9]. Recently, few abiotic stress elicitors in appropriate

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concentrations have been found very useful in this regard [10-18] that alter the biosynthetic pathway for SGs production [19]. Future challenges involve the formation of plenty of biomass by employing a liquid suspension culture technique for explant cultivation. In this respect, the measurement of physiological parameters of plant growth in liquid culture media is immensely important. On the other hand, seed quality may be difficult to obtain as very often, and there is very low germination frequency, sometimes no germination at all.

Based on our literature survey, since the *Stevia* is a photosensitive plant for the SGs accumulation, only limited studies regarding clonal propagation of S. rebaudiana with their field performance are reported. Therefore, the current study in *S. rebaudiana* aims to establish a feasible and effective tissue culture protocol for increasing the mass proliferation of nodal explants in a liquid suspension medium. Moreover, the focus of our study is a comparison of different physiological parameters and SGs of commercial significance in *in vitro* and *ex vitro* (field) plants after being harvested.

2. MATERIAL AND METHODS

S. rebaudiana seeds were obtained from an agricultural company, Polisan Tarim, Istanbul, Turkey. The washing of seeds was carried out using distilled water and 0.1 % (w/v) mercuric chloride ($HgCl_2$) to get rid of disinfectants. Then the seeds were kept for germination, after which, the leaf nodes were removed to be used as explants to perform *in vitro* experiments. Murashige and Skoog (MS) medium [20] and 3 % (w/v) sucrose was used to prepare the culture medium. The pH of the medium was adjusted to 5.7–5.8 and then 0.8 % (w/v) plant agar was added for solidification. After autoclaving all media, shoot nodes present in culture plates were incubated in a 16 h light: 8 h dark photoperiod at a relative humidity of 55–60 % and 24 ± 1 °C temperature. The stock plants were obtained for further studies.

In the meanwhile, *in vitro* cultivation was also employed using axillary shoot nodes in liquid MS medium without any PGRs. The triplicate experiment was performed and all flasks were kept in a shaking incubator at 24 ± 1 °C in 16 h/8 h photoperiod for 4 weeks. The data regarding the mean length of roots and shoots, mean number of leaves and nodes per explant, and dry and fresh biomass were recorded. Afterward, shoots were shifted to glass jars (Magenta B-cap, Sigma-Aldrich, USA) having medium and maintained in the growth room.

Plantlets with healthy roots were taken out from jars and then placed into plastic trays (12 × 9 cells) having soil: compost: vermiculite: perlite mixture (10:10:1:1 (v/v/v/v)). After acclimatization in portable greenhouse (50 × 70 × 140 cm) conditions provided with cool-white fluorescent light (50 μmol $m^{-1}s^{-1}$ irradiance) for 4 weeks, well-growing plantlets were moved to the field (200 m^2), in Gebze, Turkey to carry out field trials for 10 weeks. Field trials were conducted with 10 plants per m^2 without the application of any fertilizer.

SGs analysis was conducted using leaves from in vitro regenerated plantlets and shoots. Plant material taken out from plates and jars was cleaned with water carefully and dried in the oven for 48 h at 60 °C. Later on, dried plant material was grounded into a fine powder and each sample (20 mg) was taken in 2 mL centrifuge tube. Incubation was done at 55 °C for 15 min in an ultrasonic bath, after which, samples were centrifuged for 10 min at 12,000 rpm and 25 °C in a centrifuge machine. 0.22 μm PTFE Millipore syringe filters were used for filtering the supernatant that was later on transferred to HPLC tubes for analysis.

The standards (rebaudioside A and stevioside) were prepared by dissolving 1 mg of standard glycosides in 30 % of distilled water and 70 % of acetonitrile. Chromatography was performed with an autosampler, a binary pump solvent delivery system, and a dual-wavelength absorbance detector operating at 350 and 210 nm. The column, with 150 × 4.6 mm length and 5 μm particle size was kept warm in a column oven system at 40 °C. Finally, the isocratic flow was performed using acetonitrile and 1 % (w/v) phosphoric acid buffer mixture at a ratio of 68:32 for 20 min.

All micropropagation experiments were performed in triplicate with the randomized design. Statistical analysis of data was done using SPSS software. ANOVA determined the statistical difference and Duncan's multiple range

test calculated the significance of the difference between means ± SE value at a confidence interval of 95 %.

3. RESULTS AND DISCUSSION

Interestingly, all physiological parameters were significantly higher in the liquid medium than the nodal explant regeneration in the solid medium. Under these circumstances, several parameters including the mean length of shoots (compare 8.5 ± 1.1 and 5.0 ± 0.9 cm in green bars of Figure 1), mean length of roots (compare 1. ± 0.5 and 1.1 ± 0.2 cm in blue bars of Fig.1) and mean number of roots (4.2 ± 0.8), nodes (7.44 ± 1.5) and leaves (17.5 ± 2.6) were found to be higher than those produced from a solid medium with a mean of 1.6 ± 0.1 roots, 14.0 ± 0.8 leaves, and a mean of 6.0 ± 0.4 nodes as shown in Figure 2. In this study, it was clearly shown that shoot organogenesis in a liquid medium can prove to be very cost-efficient to produce a large number of plants and to obtain excellent yield in a bioreactor system once established. Similarly, some earlier reports on temporary immersion [21] and bioreactor systems [22] claim the efficiency of liquid culture on a large number of plantlet formations. However, tissue browning during cultivation might be a drawback due to exogenously added growth regulators and that should be overcome by a separate multiplication medium [21]. Long exposure (more than 6 weeks) to the plant tissues in liquid culture might be incorporated with morphological and physiological abnormalities due to the presence of growth regulators [22]. Although some reports resolve using less constant immersion frequencies, well-rooted shoots increased fidelity in field conditions. However, many of these efforts are not available for large-scale production because of the additional passages of callus tissue needed for clonal propagation from microshoots. In the present study, we designed an effective regeneration system with a short regeneration process without callus formation.

Fresh biomass (0.67 g) and dry biomass (0.10 g)

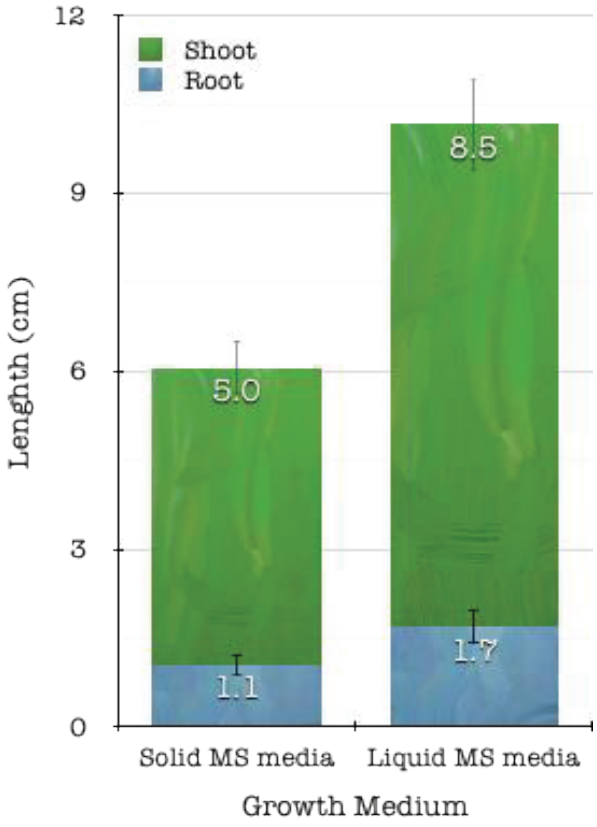

Fig. 1. Comparison of solid and liquid media in terms of shoot and root length for 4 weeks of cultivation. Bar lines represent the standard error of the mean values.

were also higher in MS liquid culturing system after 4 weeks of culturing. In this study, it was clearly shown that shoot organogenesis in a liquid medium can prove to be very cost-efficient to produce a large number of plants and to obtain excellent yield in a bioreactor system once established. Moreover, SGs content was also checked in in vitro plants. It was shown that Reb-A content (3.3 %) was significantly higher in solid MS than in liquid MS (1.4 %) medium. Similarly, ST content was almost doubled in solid MS, i.e., ST content was 0.4 % in leaves of liquid MS, much lower than 0.81 % obtained from the solid MS basal medium depicted in Figure 3. Earlier reports aiming at SGs production through the various cell and shoot cultures with or without elicitor [21, 23, 24] did not provide a reasonable amount for sustainable sweetener compound production unless in vitro raised samples were transferred progressively to ex vitro conditions [25]. Since S. rebaudiana is a photoperiod-sensitive plant, SGs accumulation is directly affected by a long day period (>14 h).

Healthy and vigorous shoots were shifted to the glass jars having MS fortified with or without rooting phytohormones, i.e., 0.25 mg/L IAA [25], and allowed to grow for 4 weeks. The percentage of rooting frequency obtained was approximately 89 %. All regenerants were healthy, therefore, shifted to about 500 jars of 450 ml volume filled with 0.4 dm^3 of soil mixture. The jars covered with the thin transparent film were placed in a growth chamber having 16 h/8 h photoperiod for 4 weeks. After hardening off in the greenhouse, almost all plants (> 98 %) were transferred to the field. Our findings revealed that all the regenerants produced in vitro were showing enough vigor to sustain the environmental conditions of the field and the average shoot length was measured to be ~ 62 cm after 10 weeks of the plantation. Figure 4 illustrates each step of the process in detail.

Table 1 shows that the Reb-A content increased from 2.6 % (w/v) in shoots to 3.7 % (w/w) in rooted-shoots, depending on the growth and development. The highest SGs content was observed after harvesting the leaf materials from the field 7.2 % (w/w). A significant difference was observed regarding ST content of 4 weeks old shoots (0.8 %, w/w) and 8 weeks old regenerants (2.0 %, w/w). Furthermore, in contrary to the rise of Reb-A content, ST content did not change and remained the same in field samples. The ratio of Reb-A and ST was obtained highest (3.6) in field-grown plants followed by 4-weeks old shoots (3.3). The ratio was lowest (1.9) in 4-weeks old

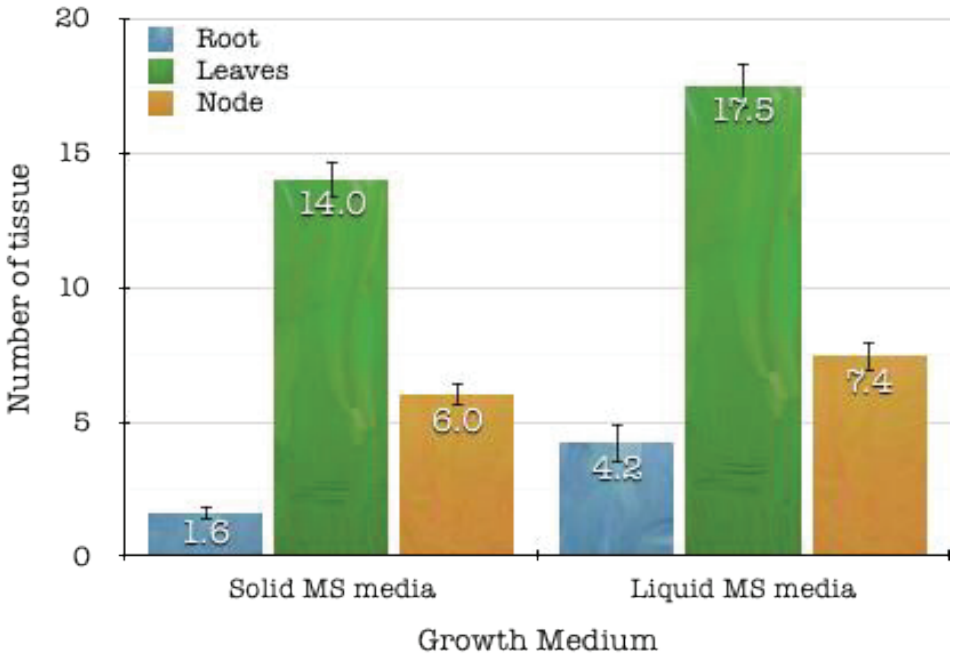

Fig. 2. Comparison of the mean number of roots, leaves, and nodes produced in solid and liquid medium for 4 weeks.

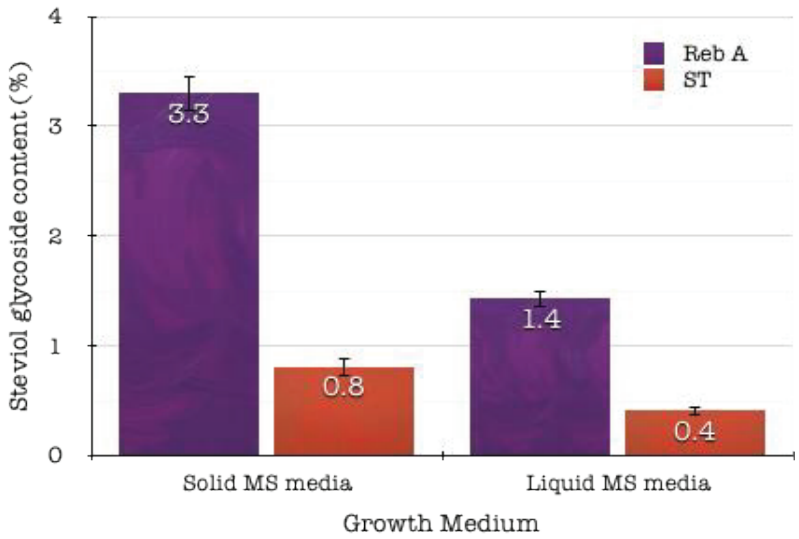

Fig. 3. Comparison of rebaudioside A (Reb-A) and stevioside (ST) content of the leaves produced in solid and liquid MS medium. Note: Bar lines represent the standard error of the mean values.

Fig. 4. Steps of tissue culture experiment conducted in S. rebaudiana from in vitro to ex vitro stages: Formation of shoots using nodes (explants) on solid MS medium for 4 weeks (A), Formation of shoots using nodes (explants) on liquid MS medium for 4 weeks (B), shoot and roots formation from liquid culture (C), root formation on MS medium having 0.25 mg/L IBA or absence of PGR (D), adventitious root formation from the shoots (E), hardening off the regenerants in jars filled with soil under plant growth room conditions (F), the general appearance of the potted regenerants one week before field plantation (G), flowering stage of the regenerants in field conditions after 12 weeks of plantation to the field (H).

Table 1. Comparison of SGs (rebaudioside A and stevioside) in the leaves of 4 weeks-old shoots, regenerants (rooted-shoots), and field samples (±: standard deviation).

Steviol glycosides	Plant Samples (%) DW*		
	Shoots (4-wk old)	Rooted-shoots (4-wk old)	Field samples (10-wk old)
Rebaudioside A	2.6±0.2[c]	3.7±0.3[b]	7.2±0.3[a]
Stevioside	0.8±0.0[b]	2.0±0.1[a]	2.0±0.1[a]
RebA/ST	3.3	1.9	3.6

* Same letter in the rows represents no significant difference at a 95 % confidence interval. Data were obtained from dried leaf materials.

regenerants due to higher ST content in them. The most probable reason might be the involvement of critical enzymatic reactions in the biochemical pathway of stevioside synthesis during root formation [26].

4. CONCLUSION

In conclusion, based on this protocol, about 32,500 healthy plantlets of ~16 cm in length can be produced within 7 months to conduct field trials. Most importantly, a significantly higher yield of *Stevia* plantlets has been obtained in MS liquid culture at a small laboratory scale. Commercial production of more SGs from these plantlets still needs further refinement of this technique by developing a suitable bioreactor system. Once an automation system is established for *Stevia* plants containing value-added SGs, it is noteworthy to mention a cost-efficient production targeting both agricultural practices and refinery systems for the natural sweeteners.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

7. DECLARATION

The results of this study are original, the same material is neither published nor under consideration elsewhere, approval of all authors has been obtained, and in case the article is accepted for publication, its copyright will be assigned to the Pakistan Academy of Sciences

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Pakistan Academy of Sciences

Research Article

Investigation of Prevalence and Awareness of Polycystic Ovary Syndrome among Pakistani Females

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Abstract: Polycystic ovary syndrome (PCOS) is a polygenic, endocrine disorder causing ovarian dysfunction. This syndrome encompasses a broad spectrum of phenotypic expression due to heterogeneity. This study aimed to investigate the prevalence and phenotypic factors contributing to the onset of PCOS in Pakistani women. Participating females (n=130) were recruited from different regions of Pakistan explicitly those who were trying to conceive for years. We designed a questionnaire having different subsections comprising questions related to psychological and environmental aspects linked with PCOS. We also analyzed the proportion of women having prior awareness about the genetic basis of the disease. The retrieved data was analyzed through SPSS V.21 by employing descriptive statistics. A Chi-square test was used to establish a correlation between PCOS and associated symptoms. Our findings suggest that factors like obesity, facial and abdomen hair growth, irregular periods, menstrual flow, cramps, and hormonal acne corresponds to PCOS in participating females with a p-value ($0.000 < 0.05$). Around half of the participants were reported to be experiencing one or other symptom related to PCOS from which hirsutism and anxiety were the most common. Furthermore, our findings indicate that multiple psychological and environmental factors contribute to the onset of the disease with a P-Value ($0.000 < 0.05$). However, a significantly higher p-value ($0.247 > 0.05$) for excessive hair loss in participants were observed demonstrating that hair loss is not linked with PCOS. In this survey, 46.5 % of participants responded that they know that PCOS can be inherited. 28.5 % of women responded that they had a family history of PCOS. Adopting a healthy lifestyle and maintaining a healthy weight can minimize the severity of PCOS. This survey evaluates different hypotheses which would facilitate a better understanding of the prevalence and associated symptoms of PCOS in Pakistan thereby enabling researchers to develop better diagnostic, management, and treatment strategies for patients.

Keywords: Pakistani, PCOS, Genetic disorder, Prevalence, Awareness, Infertility, Obesity

1. INTRODUCTION

Polycystic ovary syndrome (PCOS) is a complex disorder associated with an imbalance of hormones that causes menstrual irregularities and the appearance of cysts on one ovary or sometimes both ovaries. Affected individuals generally represent clinical heterogeneity. Commonly reported symptoms include hirsutism, acne, infertility, obesity, early onset of type 2 diabetes mellitus (DM), and alopecia [1, 2]. The prevalence of PCOS is quite higher in Pakistani women 52 % as compared to western Caucasian women, for instance, 20-25 % in the UK [3-7]. So far, the exact inheritance pattern of PCOS is unclear. However, various studies suggested that the PCOS mode of inheritance could be autosomal dominant with reduced penetrance, multifactorial and X-linked [8]. This condition develops during the early years of puberty and is determined by the interplay of a variety of genetic and environmental factors [9]. Previously considered a disorder of adult women, PCOS, in recent studies, has been stated as a lifelong syndrome, manifesting since prenatal age. The clinical presentation of PCOS changes with age, with young women complaining mostly of reproductive and psychosocial issues and elderly women complaining primarily of metabolic

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symptoms. Poor food choices and physical inactivity can increase the environmental risk of PCOS. Obesity, infectious agents, and pollutants may also play a role. With lifestyle changes including weight loss and exercise, the reproductive and metabolic characteristics of PCOS can occasionally be reversed [10].

In 1990, the National Institutes of Health (NIH) referred to hyperandrogenism and oligoovulation as a diagnostic criteria for PCOS. This results in the exclusion of other disorders mimicking PCOS such as adult-onset congenital adrenal hyperplasia, hyperprolactinemia, and androgen-secreting neoplasms [11]. The most widely used diagnostic methods including ultrasonography and hormonal tests have been considered insufficient to evaluate androgen excess, ovulation, and polycystic ovaries. So far, a 100 % authentic diagnostic method is unavailable for PCOS [12]. Also, little data is available regarding PCOS symptoms, management, and treatment options.

We investigated PCOS awareness among women and found it deficient in the general population. This study is performed to highlight the symptoms and prevalence of PCOS among Pakistani women.

2. MATERIALS AND METHODS

2.1 Data Collection

Primary research data was collected by using a questionnaire. Google forms were distributed via the internet to different internet-based platforms like WhatsApp and Instagram.

A total of 130 females aged 18-30 years from Lahore, Sialkot, Islamabad, and Gujranwala participated in this study. Data confidentiality and compliance with the Declaration of Helsinki were ensured.

The questionnaire was a structured questionnaire that had a total of 24 questions, all of which were close-ended questions. The questions targeted three different aspects related to PCOS and hence the questionnaire was divided into three different sections.

The 1st section consists of questions that helped gain primary data on whether or not the females facing symptoms of PCOS were aware of having a disease or not. The questions were based on associated clinical symptoms of PCOS such as obesity, excessive hair growth on the face and lower abdomen, painful and heavy blood flow, irregular periods, hormonal acne, and hair loss leading to bald spots.

The 2nd section of the questionnaire contained questions related to mood swings, low self-esteem, depression, body image, eating disorders, anxiety, getting tired easily, and having trouble conceiving.

The 3rd section of the questionnaire consisted of whether the participants were aware of the fact that PCOS can be inherited.

2.2 Statistical Analysis

Inferential statistics were used to determine the association of symptoms with PCOS. The data was analyzed using SPSS V21. Received data from the questionnaire was carefully recorded in SPSS spreadsheet. The correlation was established by using chi-square. Statistical significance was defined as $P<0.05$.

3. RESULTS

All participating women (n=130) were in their reproductive ages (18-30 years); having at least higher secondary education; trying to conceive for years recruited for this study (Fig. 1A).

Firstly, we performed a phenotypic analysis of PCOS symptoms such as for overweight, excessive hair growth on the face and lower abdomen, painful periods and heavy blood flow, irregular periods, hormonal acne, and hair loss leading to bald spots (Fig. 1B & 1C). Correlation values to the symptoms are shown in (Table 1).

Our results exhibit that all these symptoms correspond to PCOS among participants with a p-value ($0.000 < 0.05$). Hence, we reject the null hypothesis and conclude that there is a significant relationship between these symptoms causing PCOS in individuals.

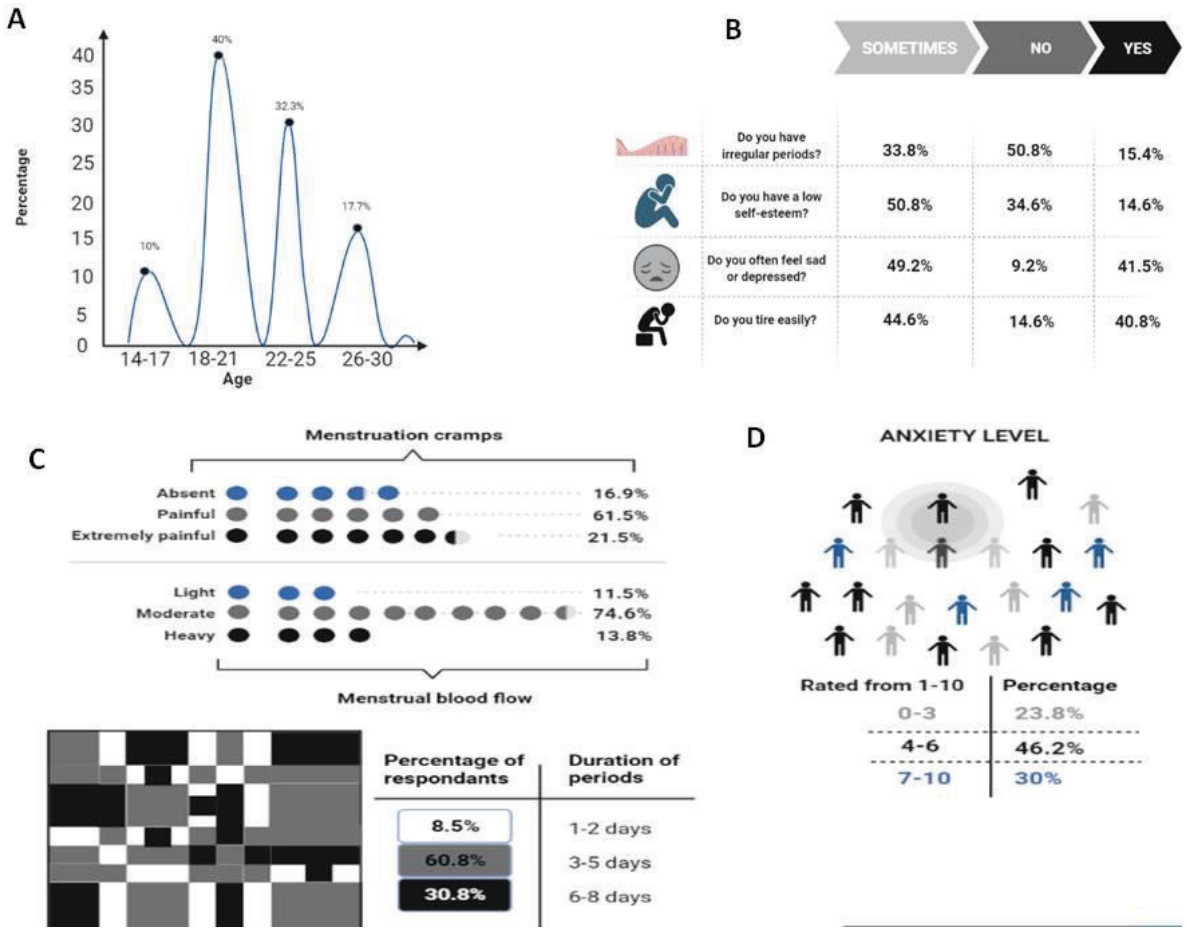

Fig. 1. Pictorial representation of different factors associated with PCOS. (A) Graph indicating ages of participating females; B. Chart showing responses received for questions related to the level of self-esteem, regularity of periods, and tiredness; C. Pictorial representation of responses received for menstrual cramps (Higher); D. High Level of anxiety in the participants.

However, the p-value of excessive hair loss in individuals (0.247 > 0.05) is significantly higher hence, it is quite considerable that there is no association between hair loss and PCOS. Referring to the data obtained through the survey which shows that 50 % of our respondents agreed that they face issues of extreme hair loss which is contradicting the statistical results (Fig. 2A). This explains that the combination of various symptoms may cause extreme loss of hair in some individuals.

Secondly, questionnaire section 2 comprised of psychological and environmental aspects which have a direct relation to the occurrence of PCOS. The Chi-square test was used again to understand the correlation between PCOS and the psychological and environmental aspects. The aspects for investigation were mood swings, self-esteem level, depression, body image, eating disorder, obesity anxiety, getting tired easily, and having difficulties conceiving as shown in (Fig. 2B & 1D). Results showed that the p-value of all these factors corresponds to PCOS (0.000 < 0.05) hence, we reject the null hypothesis and conclude that there is a significant relation between these psychological and environmental factors with PCOS (Table 2).

Thirdly, our area of focus was to investigate whether the participants were aware of the fact that PCOS can be inherited (section 3). Studies have proven polycystic ovary syndrome to be a familial condition but do not have a clear pattern of inheritance. In this survey, 46.5 % responded that they know that the disease can be inherited (Fig. 3A). In this survey, 28.5 % of individuals responded with “yes”, and agreed that they had affected females in their families.

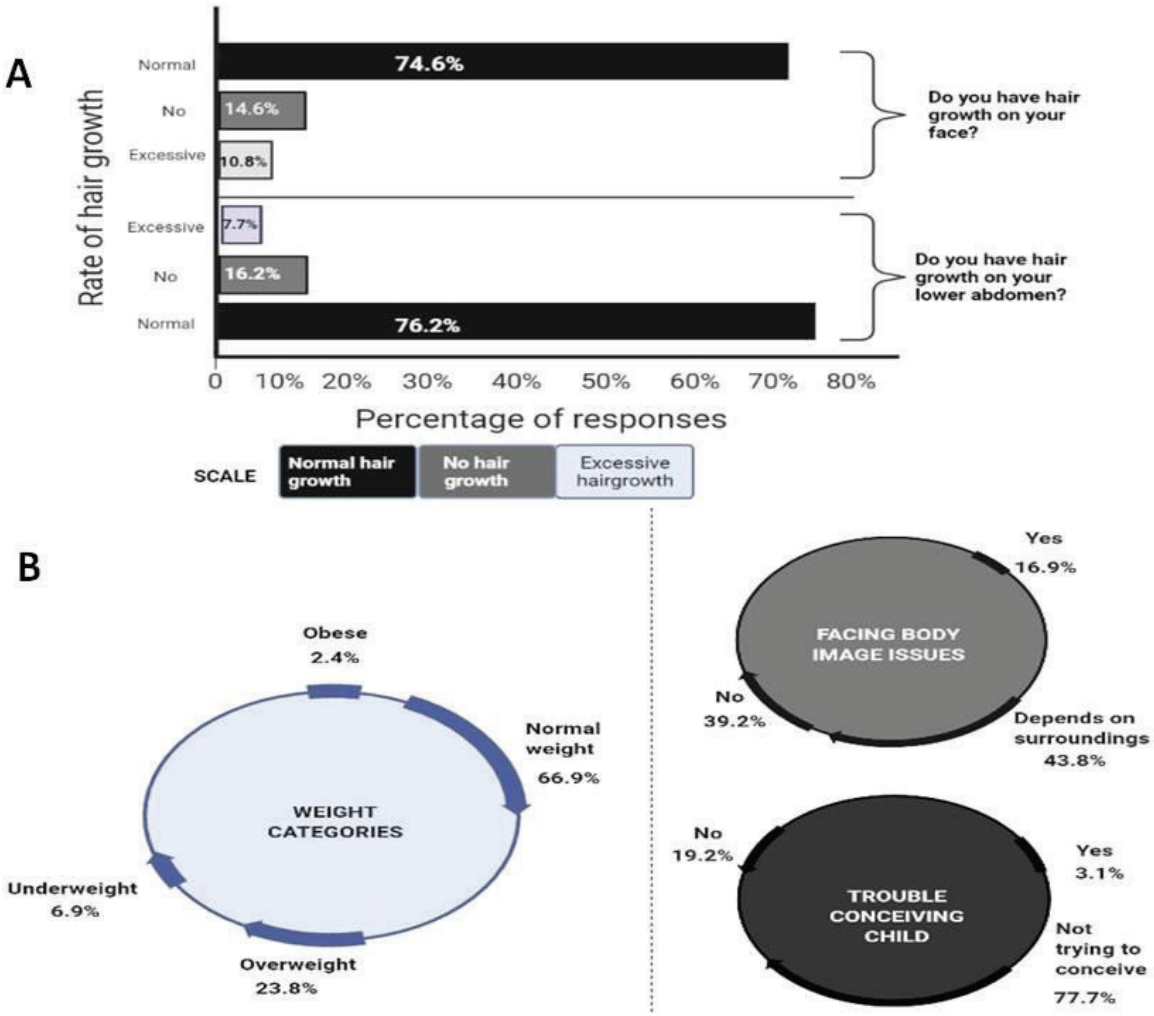

Fig. 2. Cartonic representation of different factors associated with PCOS and responses received from participants. A. Proportion of individuals suffering from extreme hair loss; B**.** The proportion of individuals who had weight issues and trouble conceiving a child.

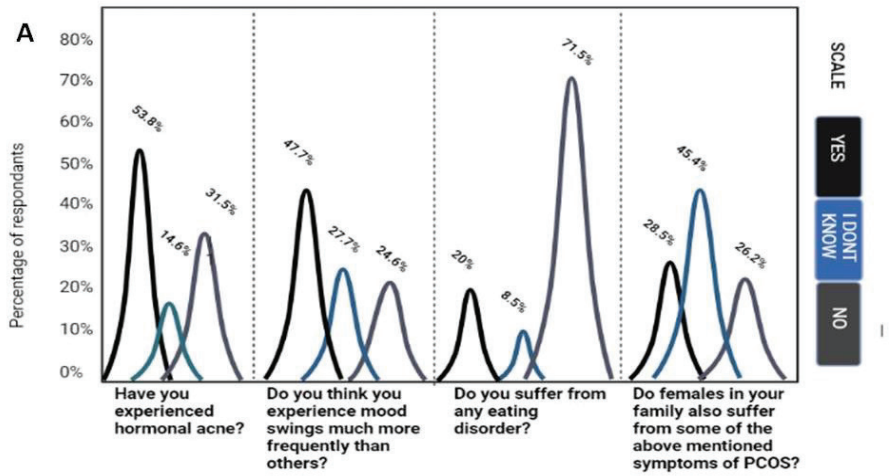

Fig. 3. Graphical representation of multiple responses linked to PCOS

Table 1. Chi-square test to identify the correlation between the symptoms and PCOS

	Weight	Hair Growth on Face	Hair Growth on Lower Abdomen	Menstrual Blood Flow	Irregular Periods	Menstrual Cramps	Hormonal acne	Extreme hair loss	Bald Spots
Chi-Square	300.446[a]	236.041[b]	244.868[b]	239.736[b]	54.579[b]	103.570[b]	69.157[b]	1.339[c]	175.35[c]
df	3	2	2	2	2	2	2	1	1
Asymp. Sig.	.000	.000	.000	.000	.000	.000	.000	.247	.000

*Df degrees of freedom; Asymp. Sig. Asymptotic Significance

Table 2. Chi-square test indicating the correlation between the psychological and environmental factors and PCOS

	Mood Swings	Low Self Esteem	Depression	Body Image	Eating Disorder	Anxiety	Fatigue	Trouble Conceiving
Chi-Square	5.474[a]	71.289[a]	71.158[a]	57.579[a]	239.474[a]	51.026[a]	38.000[a]	197.053[a]
df	2	2	2	2	2	2	2	2
Asymp. Sig.	.065	.000	.000	.000	.000	.000	.000	.000

*Df degrees of freedom; Asymp. Sig. Asymptotic Significance

4. DISCUSSION

Polycystic ovarian syndrome (PCOS) is among one of the most debatable endocrine disorders with no exception in Pakistan. It is one of the major causes of infertility in females with little awareness of its symptoms characterized by cystic acne, hair loss or baldness, obesity or having problems with weight loss, and menstrual irregularities along with heavy blood flow, among women.

In this study, a total of 130 women participated with the majority of women aged 18-30. Our results suggested that (18.4 %) of patients with PCOS have excessive facial and lower abdomen hair growth. Patients also suffer from hormonal acne (53.8 %) and body image issues (16.9 %) developed due to difficulty in weight loss and obesity. Different studies report that obesity is not a reason behind PCOS. However, obesity does aggravate many other aspects of the phenotype, especially cardiovascular risk factors such as glucose intolerance and dyslipidemia as compared to non-obese women [13-15]. These symptoms affect the quality of life of women leading to a higher prevalence of depression. Obesity has been largely associated to be the major factor causing depression and emotional stress in this study. (41.5 %) participants responded that they have undergone depression and (30 %) agreed that they suffered from anxiety. The reason could be abnormal androgen production, demoralization faced by patients having higher BMI, and social withdrawal due to PCOS. A complex lifestyle stigmatizes women leading to low self-esteem as they think that they have lost their feminism [16, 17].

PCOS has a genetic basis as well as it can be inherited from one generation to the other. Many candidate genes have been found to be associated with PCOS such as *CYP11a*, *CYP21, CYP17*, and *CYP19* play an important role in causing PCOS however, psychological and environmental aspects also play a significant role in the manifestation of the disease. According to an estimation, there exists a 20 % to 40 % chance of developing polycystic ovary syndrome if the individual has an affected mother or sister. However, the role of various environmental factors is undeniable. Our findings show that 28.5 % of women responded

that their family members also suffer from PCOS. It is also supported by other studies which found the prevalence of PCOS in first-degree relatives is 55-60 % showing that it has an autosomal dominant pattern of inheritance [18].

A similar study was conducted in Pakistan to evaluate the clinical manifestation of PCOS symptoms among women. It showed a high percentage of PCOS symptoms (obesity 80%, irregular periods 71.8 %, and acne 67.3 %) therefore supporting the results of our study (irregular periods 49.2 %, acne 53.8 %, extreme hair loss 50 %, and trouble losing weight 42 %) [7].

Our study has shown that there is a high percentage of women showing symptoms of PCOS in Punjab Pakistan. The study also showed that 45.4 % of women were unaware of these symptoms being present in their family and 35.7 % do not know about their genetic prevalence. However, the sample size is a limiting factor in this study. More participants should be recruited and a pilot study can be conducted to confer the actual prevalence of PCOS in Pakistan.

Adopting a healthy lifestyle and reducing weight can reduce the severity of PCOS. The progression of PCOS can also be reduced through having a balanced and healthy diet that is low in fat with rich in fiber. Hence there is a need to create an awareness regarding the symptoms of PCOS so that it can be treated accordingly and women can lead a good quality of life.

5. CONCLUSION

PCOS is a complex condition highly prevalent in Pakistani women. Prevention by the rapid diagnosis of at-risk pre-pubertal and early pubertal girls through lifestyle modifications is a future aim. The need for long-term studies is crucial to understand which phenotypes will present additional health risks at increased age and if there is a difference in morbidity rates among PCOS patients. Advanced techniques like Genome-wide association studies (GWAS) can be employed to recognize and track the genetic spectrum of this condition. Timely diagnosis could be a remarkable breakthrough in the management and treatment. In treatment modalities, assisted reproductive technology (ART) could be opted for treating infertility in PCOS patients. Public awareness seminars can also help in educating women about this condition. There is a need to do more research on the genetics and etiology of PCOS for anticipation of threats and treatment paradigms of this syndrome.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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