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COMPARATIVE STUDY OF GROWTH AND BIOCHEMICAL ATTRIBUTES OF *AVICENNIA MARINA* (FORSSK.) VIERH OF INDUS DELTA AND *IN VITRO* RAISED PLANTS

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Abstract

The study was conducted to optimize medium and growth conditions for micropropagation and field establishment of grey mangrove, *Avicennia marina* (Forssk.) Vierh using nodular stem sections as explants. Varying concentrations of 6-benzylaminopurine (BAP), kinetin (Kin) and indole-3-acetic acid (IAA) were supplemented in medium to assess their effects on the development of microshoots while indole-3-butyric acid (IBA), 1-naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) were supplemented to assess their role in root formation. All the treatments promoted shoot formation, the highest percentage of explants (86%) formed microshoots from the axillary buds with the highest number of microshoots per explant (2.4±0.1) were noted on MS medium consisting of 0.5mg/L of BAP, 1.0mg/L of Kin and 0.25mg/L IAA. In contrast 1.0mg/L IBA containing medium caused root formation in the maximum microshoots (82%) with 2.16±0.05 roots per microshoot, 27.5±0.5mm average root length. Furthermore, 65% of micropropagated *A. marina* plants were successfully survived for two years during 2015-17 in region II (Jamshoro). These plants showed better growth as plant height, numbers of leaves, chlorophyll contents, sugars and proteins were increased comparing with same age plants of region I. On the other hand, an increase in stem diameter, number of branches, and the amount of total phenolic contents, total flavonoids and antioxidants was found in plants of region I (Shah Bander, Indus Delta). In conclusion, micropropagated plantlets of *A. marina* were successfully established in geographically hot region which will support to establish mangrove plants around the banks of Indus River.

Key words: *Avicennia marina*, Micropropagation, Chlorophyll contents, Carbohydrates, Proteins, Phenolic acids.

Introduction

Mangroves are a diverse group of plants found in the coastlines of tropical and subtropical intertidal zones of 118 countries and territories. Mangroves cover an area of above 18 million hectares of the globe representing 42% in Asia and about 7% in South Asia (Giri *et al*., 2015). Pakistan ranked to have 24th largest mangrove forest and 95% of mangroves forests are found in the marshes of Indus Delta of Sindh province along the coastlines of Arabian Sea (Giri *et al*., 2015). *A. marina*is the predominant mangrove species of Indus Delta covering about 97% of mangrove forests together with *Aegicerias corniculata*, *Ceriops tagal* and *Rhizophore mucronata* (Aziz & Khan, 2000; Giri *et al*., 2015). The existence of these mangrove forests are permanently dependant on supply of fresh water and residues from River Indus that flows through Indus Delta to Arabian Sea.

A. marina of family 'Avicenniaceae' is a mangrove tree that can attain above ten meters height (Giesen *et al*., 2007). *A. marina* is a multi-use tree but in Pakistan its utilization is restricted to few types such as wood and fodder by coastal populations (Spalding *et al*., 1997; Tariq *et al*., 2006). *A. marina* plant is rich source of alkaloids, steroids, triterpenes, saponins, flavonoids and tannins that possess pharmacological, toxicological, and ecological importance (Molaeea *et al*., 2017). The aerial parts of *A. marina* possess antiviral, antiparasitic, antifungal, antibacterial, anti-cancer anti-ulcer activities (Bandaranayake, 2002; Khafagi *et al.*, 2003; Arivuselvan *et al*., 2011; Iranawati *et al*., 2018; Yang *et al*., 2018).

Mangrove habitat is severely destructed due to land reclamation and industrial effluents (Kathiresan & Bingham, 2001) with estimated global mangrove loss of about one million hectares each year (Mohamed, 1996). The Indus Delta is one of the most threatened among large deltas due to utilization of upstream freshwater for agriculture. The sever reduction in upstream flow of fresh water increased the concerns about conservation of diversity in mangrove habitat.

There is an urgent need of mangrove afforestation program for conservation of habitat and to increase the productivity of intertidal zones of the coast, the deltas and banks of major rivers systems. Mangrove species are mostly regenerated through seeds but there are problems of collection of propagules from natural environment, post dispersal predation of seed by crab, short life of seed viability and seed storage as most seeds are of large size (Robertson *et al*., 1990; Saenger & Siddiqi, 1993). Due to dynamic nature of mangrove habitat, the survival of seedlings in the potential areas is also poor (Saenger & Siddiqi, 1993). There is a necessity of an alternative to seed regeneration as large scale *In vitro* propagation for prepetuation of mangrove species and their reestablishment in the field (Hartmann & Kester, 1989). Mangrove tissue culture and *In vitro* micropropagation technology can be used as an alternative for the conservation of unique genotypes for commercial purpose. Tissue culture of mangroves is difficult because of explants which turn brown and die after *In vitro* culturing (Kathiresan & Bingham, 2001). Only few mangrove species have been micropropagated successfully such as *Excoecaria agallocha*, *Avicennia officinalis* and *Avicennia marina* (Al-Bahrany & Al-Khayri, 2003: Arumugam & Panneerselvam, 2012).

Jamshoro city is geographically located on 25.45°N, 68.28°E on right bank of Indus River at a distance of about 18 km far from Hyderabad, 150 km from Karachi and 180 km from Shah Bunder. The average high temperature in Jamshoro is 40.2°C during summer and average low temperature is 12.4°C during winter. The present study was planned to micropropagate and to have more insight into some growth and biochemical attributes of *A. marina* plants growing in intertidal zones of Indus Delta and *In vitro* raised plants growing in the new environment of Jamshoro. This is first report describing micropropagated plantlets of *A. marina* established in a geographically different location (Fig. 1).

Material and Methods

***In vitro* micropropagation of *A. marina*:** The shoots of 2–3 years old *A. marina* plants were collected from provenances of creeks of Shah Bunder, district Thatta, Sindh during August - November 2014 (Fig. 1). The leaves were removed and about 8-10 cm nodal stem sections were cut, washed with tap water for 30 minutes and then dipped in 70% ethanol for one minute followed by in 1.5% (w/v) sodium hypochloride with 3 drops of tween 20 for 15 minutes under shaking conditions (Peiris *et al*., 2012). Finally the explants were rinsed three times in sterile distilled water (5 min per rinse) and cut into smaller (2–2.5 cm) nodal sections. The explants were inoculated on semi solid medium in baby jars and incubated in growth room at 25±2°C under a 16 hours photoperiod provided by white florescent tube lights (2500 lux). The shoot formation medium contained MS medium (Murashige & Skoog, 1962) supplemented with 100mg/L myo-inositol, 2.0mg/L glycine, 6.0g/L activated charcoal, 150mg/L ascorbic acid, 3.0% sucrose, 0.8% agar and various concentrations of BAP, Kin and IAA. The pH of the medium was adjusted to 5.8 with 0.5N NaOH, boiled in microwave oven to melt agar, dispensed in baby jars (30ml/jar) and sterilized in autoclave at 121°C for 15 min. The explants that formed shoots were subcultured six times on same fresh media after every fifteen days for three months.

***In vitro* root formation and field establishment of *A. marina*:** *In vitro* raised microshoots (2-4 leaves stage) were excised from original explant, transferred on rooting medium and incubated in the same environmental conditions. The rooting medium consisted of MS medium additionally supplemented with 100mg/L myo-inositol, 2mg/L glycine, 4.0g/L activated charcoal, 100mg/L ascorbic acid, 3.0% sucrose, 0.8% agar and various concentrations of IBA, NAA and IAA. The root length was measured after 12 weeks of transfer on rooting media at the time of shifting in soil. The plantlets that formed roots were carefully shifted in pots containing garden soil and peat moss (1:3). The pots were covered with transparent plastic to maintain humidity and acclimatized in growth room at 26±2°C (Bahrany & Al-Khayri, 2003). The plastic covering was gradually removed after two weeks and then pots were shifted in green house for further growth. After three months, plants were shifted in the field (region II) and the data for various growths attributes such as survival of plants during field acclimatization, plant height, stem diameter, number of shoots per plant and number of leaves per micropropagated plant was noted for successive two years during 2015-17. The growth attributes were also compared with *A. marina* plants propagated through propagules at Mangrove nursery established by WWF and Sindh Forest Department at Shah Bander, district Thatta (region I).

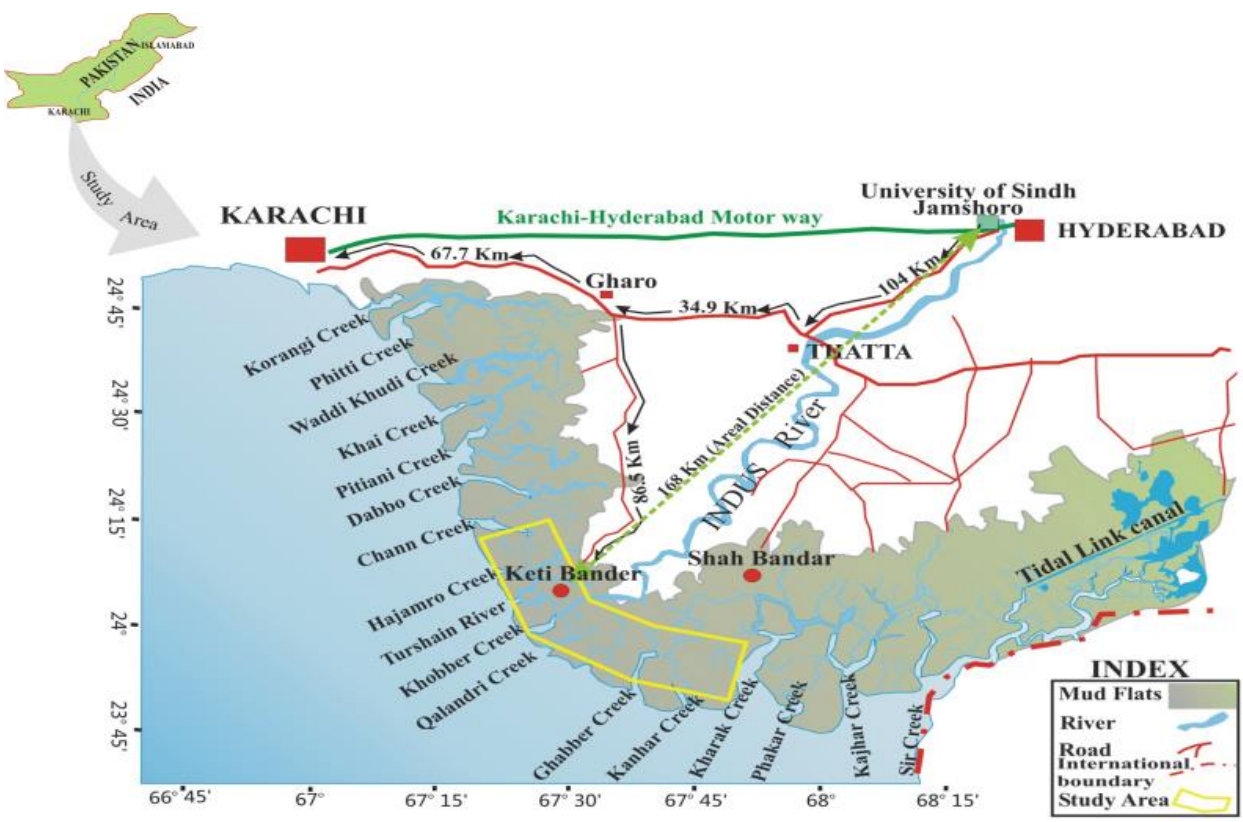

Fig. 1. Geographic location of study area showing region I (Shah Bander) and region II (University of Sindh, Jamshoro (Saied *et al*., 2013).

Determination of chlorophyll contents: Chlorophyll and carotenoids in second leaf from top of the *A. marina* plants of Shah Bander (region I) and micropropagated plants established at Jamshoro (region II) were quantified through spectrophotometer following reported method and calculated according to formulae (Lichtenthaler & Wellburn, 1983).

$Ca = 11.75\ A_{662} – 2.350\ A_{645}$
$Cb = 18.61\ A_{645} – 3.960\ A_{662}$
$C_{x+c} = 1000\ A_{470} – 2.270\ Ca – 81.4\ C_b/227$
Total Chlorophyll = Ca + Cb

where A is absorbance, Ca is chlorophyll *a*, Cb is chlorophyll *b*, and C_{x+c} is total carotenoids [xanthophyll (*x*) plus carotenes (*c*)].

Extraction of biochemicals and their quantification: The root, bark and leaf samples were collected from two years old *A. marina* plants growing at Shah Bander (Region-I) and *In vitro* raised plants established in field at Jamshoro (Region-II). The samples were dried in oven at 40°C for 72 hours. 10% solvents extracts of root, bark and leaf explants were prepared separately in 70% methanol, 70% ethanol, 70% acetone and water following standard method. Total soluble sugars in extracts of *A. marina* from region I and II were analyzed using standard spectrophotometric method (Montgomery, 1961) using glucose as standard and reducing sugars by Miller (1959) method using glucose as standard. Total proteins were quantified by Lowry *et al*., (1951) method using bovine serum albumin as the standard. Similarly total phenolic acids were determined by Folin-Ciocalteu method (Singleton & Rossi, 1965) and the results were expressed as Gallic acid equivalent per gram dry weight of sample (GAE/g dw). Total flavonoids were quantified by Chang *et al*., (2002) method using rutin as standard flavonoids and antioxidant capacity by Prieto *et al*., (1999) method using α - tocopherol as standard.

Statistical analysis

The data was collected in triplicate, the data was collected as mean of triplicates and standard deviation was calculated by using Microsoft excel 2010. The analysis of variance (ANOVA) was done showing the *p*-value (0.05).

Results and Discussion

***In vitro* micropropagation of *A. marina*:** The axillary buds in nodal sections have potential to develop into whole plant when cultured on suitable nutrient media and plant growth regulators. In present study, the nodal stem sections of *A. marina* were successfully micropropagated into microshoots on MS media supplemented with variable concentrations of BAP, Kin and IAA. According to results (Table 1), 27–76% of the explants were proliferated into microshoots on MS media additionally supplemented with 0.5–2.0 mg/L of each BAP and Kin separately. The addition of cytokinin – auxin in combination in MS medium increased the efficiency of microshoot formation up to 86%. In all the treatments 1.2±0.05 – 2.4±0.1 microshoots per explant were formed with 11.5±0.5 – 25.0±0.45 mm shoot length within 45 days of inoculation. MS medium supplemented with 0.5mg/L BAP, 0.25mg/L IAA and 1.0mg/L Kin showed 86% shoot proliferation with 2.4±0.1 microshoots per explant and 25.0±0.45 mm shoot length within 45 days of culture (Table 1). Our results showed that addition of 0.5mg/L BAP, 0.25mg/L IAA and 1.0mg/L Kin in combination in media was more effective in inducing proliferation of microshoots, multiple shoots formation with significant increase in shoot length.

Micropropagation of plants is not expensive but its success is limited in mangroves because the explants exude phenolic acids, frequently turns the explants brown and eventually dies during *In vitro* culture (Kathiresan & Ravikumar, 1997). Only few mangroves had been micropropagated successfully such as *Avicennia marina* (Al-Bahrany & Al-Khayri, 2003), *Excoecaria agallocha* (Arumugam & Panneerselvam, 2012) and *Sessuvium portulacastrum* (Kathiresan & Ravikumar, 1997). In present study, nodal segments of *A. marina* showed good morphogenetic response of microshoot formation on MS media supplemented with BAP, Kin and IAA in combination. Auxin and cytokinin in combination may control the morphogenetic responses. Arumugam & Panneerselvam (2012) also reported the combined action of BAP with NAA on *In vitro* axillary shoot multiplication in *Excoecaria agallocha*. Al-Bahrany & Al-Khayri (2003) reported single shoot formation from one or both preexisting axillary buds in nodal stem sections while we obtained up to 2.4±0.1 microshoots per explant. Similarly, Al-Bahrany & Al-Khayri (2003) used 1.0mg/L kin and 0.5 mg/L BA for optimum shoot formation while in present study the best results of shoot formation was obtained on MS media supplemented with 1.0mg/L kin, 0.5 mg/L BA and 0.25 mg/L of IAA. Furthermore, it was observed that the higher concentrations of BAP and Kin significantly decreased the microshoot formation in *A. marina*. In agreement with the previous reports, current study reveals the positive response of shoot formation attained by using low concentration of IAA in combination with BAP and Kin.

***In vitro* root formation and field performance of *A. marina*:** According to results shown in table 2, the percentage of microshoots rooted and number of roots per microshoot were significantly varied in MS media supplemented with different concentrations of IBA, NAA and IAA. IBA showed the most effective response in root formation in comparing with IAA and NAA. The results revealed that most of the microshoots commenced root formation during 6-8 weeks of transfer on rooting media. MS medium supplemented with 1.0mg/L IBA showed the highest response as 82% microshoots were rooted with 2.16±0.05 roots per shoots and 27.5±0.5 mm of average root length on 43rd day of transfer on rooting media (Table 2). The efficiency of root formation, root length,

and number of roots per microshoot were decreased as IBA concentrations were manipulated singly or supplemented in combinations with NAA and IAA. Furthermore, microshoots were failed to develop roots when inoculated on simple MS medium without any plant growth regulator (Table 2). Al-Bahrany & Al-Khayri (2003) obtained 2.2 roots per shoot using 4.0 mg/L of IBA in ten weeks but in current study 2.28 roots per microshoot was achieved using 0.5mg/L IBA in seven weeks. Alatar (2015) also obtained roots in *Rauvolfia serpentine* through using IBA. In current study, 73% microshoots were also rooted with 1.8±0.05 roots per shoots and 23.2±0.75 mm of average root length was obtained on media containing 1.0 mg/L IBA and 0.5 mg/L NAA in combination. Geetha *et al*., (1997) also reported root formation in microshoots of *Kaempferia* spp., in media containing IBA and NAA in combination. Husen & Pal (2007) reported that auxins (IBA or NAA) enhances the soluble sugars and starch levels in rooting zones of shoot cuttings which are utilized as energy source for cell division and differentiation in the zones.

In current study, *In vitro* raised plantlets were successfully hardened in pots containing sterile garden soil for four weeks and then established in green house. It was noted that 65% micropropagated plants established in region II (Jamshoro) were successfully survived during hot summer (about 45°C, 30-70% relative humidity) and cold winter (about 05°C). The slow growth was noted during hardening and field establishment of micropropagated plantlets however, significant increase in plant height was noted after 16 weeks. The growth of *A. marina* plants of region II was noted after 24 months and compared with same aged plants naturally growing in region I. According to obtained data (Table 3), micropropagated *A. marina* plants established in region II (Jamshoro) showed increased stem height and number of leaves but decreased stem diameter and number of shoots comparing with same age plants naturally growing in region I (Shah Bander).

Chlorophyll contents in *A. marina*: In present study total chlorophyll contents and carotenoids were analyzed during different months of the year. The results showed significant ($p<0.05$) variation in chlorophyll contents in micropropagated *A. marina* plants in region II which was lesser in plants of region I (Table 4). The highest amount of total chlorophyll contents (3.96±0.12 mg/g of fresh wt) and carotenoids (0.71±0.12 mg/g of fresh wt) and lesser chl *a/b* ratio (1.95) was found in the plants at region II during the month of October. On the other hand, a decreased amount of total chlorophyll contents (2.99±0.14 mg/g of fresh wt) and carotenoids (0.42±0.05 mg/g of fresh wt) was found in the plants at region II in the month of January with higher Chl *a/b* ratios (2.57 and 2.47) during the months of April and January respectively (Table 4). In case of region I, overall total chlorophyll contents and carotenoids are lesser than region II. This may be due *A. marina* plants in region I were grown in sea water (highly saline water) but for growth of plants in region II river water (sweet water) was used.

Table 1. Effect of different concentrations of BAP, Kinetin (Kin) and IAA on *In vitro* shoot formation in *A.marina*.

MS medium with PGRs (mg/L)			% of explants formed shoots	No of shoots per explant (Mean+SD)	Average shoot length in mm after 45 days (Mean+SD)
BAP	IAA	Kin			
0	0	0	0	0 K	0 M
0.5	0	0	56	1.2 ± 0.06 J	13.8 ± 0.76 J
1.0	0	0	72	1.6 ± 0.06 GH	19.0 ± 0.5 FG
1.5	0	0	52	1.4 ± 0.12 I	14.5 ± 0.5 J
2.0	0	0	27	1.2 ± 0.06 J	12.5 ± 0.5 K
0	0	0.5	58	1.2 ± 0.06 J	16.2 ± 0.85 I
0	0	1.0	76	2.0 ± 0.17 BC	19.6 ± 0.85 EF
0	0	1.5	71	1.6 ± 0.05 GH	20.5 ± 1.0 DE
0	0	2.0	45	1.2 ± 0.06 J	11.5 ± 0.5 L
0.5	0.25	0.5	73	1.5 ± 0.05 HI	22.8 ± 0.3 B
0.5	0.5	0.5	63	1.4 ± 0.08 I	18.0 ± 0.5 H
0.5	1.0	0.5	38	1.2 ± 0.05 J	16.5 ± 0.5 I
0.5	0.25	1.0	86	2.4 ± 0.1 A	22.5 ± 0.75 B
0.5	0.5	1.0	83	2.1 ± 0.1 B	25.0 ± 0.45 A
0.5	1.0	1.0	56	1.6 ± 0.06 FG	21.2 ± 0.25 CD
1.0	0.25	0.5	78	1.8 ± 0.08 DE	18.2 ± 0.3 GH
1.0	0.5	0.5	72	1.6 ± 0.1 GH	21.5 ± 0.5 C
1.0	1.0	0.5	43	1.2 ± 0.05 J	14.7 ± 0.3 J
1.0	0.25	1.0	78	1.9 ± 0.12 CD	18.7 ± 0.3 FGH
1.0	0.5	1.0	76	1.7 ± 0.06 EF	19.0 ± 1.0 FG
1.0	1.0	1.0	14	1.2 ± 0.05 J	12.8 ± 0.25 K

Table 2. Effect of different concentrations of IBA, NAA and IAA on root formation, root length and days to root formation in *A. marina*.

MS Medium with PGRs (mg/L)			% of microshoots rooted	No of roots per microshoot (Mean+SD)	Average root length in mm (Mean+SD)	Days to root formation (Mean+SD)
IBA	NAA	IAA				
0	0	0	0	0 ± 0 I	0 ± 0 K	0 k
0.12	0.0	0.0	09	1.16 ± 0.07 H	12.6 ± 0.57 H	56.0 ± 2.8 C
0.25	0.0	0.0	29	1.35 ± 0.1 F	15.5 ± 0.5 F	50.7 ± 1.53 G
0.5	0.0	0.0	71	2.28 ± 0.1 A	22.3 ± 0.65 D	40.0 ± 1.0 J
1.0	0.0	0.0	82	2.16 ± 0.05 B	27.5 ± 0.5 A	42.7 ± 1.53 I
1.5	0.0	0.0	64	2.16 ± 0.1 B	25.4 ± 1.01 B	43.7 ± 1.5 I
2.0	0.0	0.0	31	1.34 ± 0.1 F	12.7 ± 0.7 H	54.0 ± 2.0 D
0.0	0.25	0.0	23	1.34 ± 0.1 F	14.3 ± 0.61 G	51.3 ± 1.5 FG
0.0	0.5	0.0	41	1.67 ± 0.07 D	18.95 ± 0.64 E	50.67 ± 2.1 G
0.0	1.0	0.0	22	1.5 ± 0.05 E	18.47 ± 0.5 E	53.0 ± 1.4 EF
0.0	1.5	0.0	16	1.3 ± 0.1 F	10.5 ± 0.5 J	50.0 ± 2.0 A
0.0	0.0	0.25	0	0 ± 0 I	0 ± 0 K	0 K
0.0	0.0	0.5	0	0 ± 0 I	0 ± 0 K	0 K
0.0	0.0	1.0	13	1.26 ± 0.06 G	10.8 ± 0.57 J	58.3 ± 1.53 B
0.0	0.0	1.5	11	1.32 ± 0.03 F	11.5 ± 0.43 I	55.3 ± 0.56 C
0.25	0.5	0.0	31	1.66 ± 0.1 D	12.3 ± 0.35 H	52.0 ± 2.0 EF
0.5	0.5	0.0	52	1.8 ± 0.07 C	15.5 ± 0.5 F	48.3 ± 1.5 H
1.0	0.5	0.0	73	1.8 ± 0.05 C	23.2 ± 0.75 C	48.5 ± 3.54 H
0.25	0.0	1.0	22	1.67 ± 0.1 D	11.7 ± 0.75 I	52.0 ± 2.0 EF
0.5	0.0	1.0	46	1.8 ± 0.1 C	14.5 ± 0.5 G	52.7 ± 2.1 E
1.0	0.0	1.0	61	1.8 ± 0.05 C	18.7 ± 0.76 E	51.3 ± 1.5 FG
1.0	0.5	1.0	43	1.33 ± 0.1 F	11.3 ± 0.41 I	51.3 ± 1.5 FG

Average root length was measured after 10 weeks of subculturing on rooting media

Mangroves are exceptionally dynamic plants that can survive in high salt conditions through adapting various anatomical and physiological characteristics. Therefore, factors affecting photosynthesis such as temperature, salt and geographical locations are little understood (Ball & Farquhar, 1984). In current study a total chlorophyll contents and carotenoids in leaves of *A. marina* of region II were significantly decreased during January. This may be due to decrease in temperature in region II. The growth of mangroves is limited to optimal low as well high temperatures and photosynthesis of most mangrove species declines if temperature exceeds 35°C (Moore *et al*., 1973). Kao *et al*., (2004) also observed significant reduction in chlorophyll contents in leaves of *A. marina* during low temperature at 15°C and higher than 30°C. The current findings about changes in synthesis of carotenoids in *A. marina* during different weather conditions are in agreement with Falqueto *et al*., (2008) who reported the enhanced synthesis of carotenoids in *R. mangle* and *L. racemosa* during dry and rainy seasons.

Biochemical findings: The biochemicals attributes analyzed from the leaves, bark and roots of *A. marina* plants of region I and II are presented in Fig. 2a,b and c.The results revealed that overall total soluble sugars, reducing sugars and proteins in various solvent extracts of root, bark and leaf of *A. marina* of region I1 were significantly higher ($p<0.05$) then region I. The findings showed a significantly ($p<0.05$) higher amount of total carbohydrates (118.65±4.5mg/ml) and total proteins (55.49±2.5mg/ml) in ethanol extracts and reducing sugars (62.41±3.12mg/ml) in methanol extracts of leaf of *A. marina* of region II as compared to other solvent extracts of *A. marina* of region 1 and II (Fig. 2a,b and c). To regulate metabolism under stress environment, plants synthesize and accumulate compatible solutes such as sugars, proteins, amino acids, etc. According to Prado *et al*., (2000) sugar contents in different parts of plants are enhanced under different environmental stresses. Parida *et al*., (2004) reported that the accumulated sugar and starch under salt stress may play role in osmoprotection, carbon storage and radical scavenging. In present study total soluble sugars and reducing sugars in various parts of *A. marina* were significantly higher in region II while lower in *A. marina* plants of region I. Similarly mangroves have adopted some physiological mechanisms such as salt excretion and ultra filtration at root level to deal with salts stress conditions (Drennan & Pammenter, 1982), so salt stress may not enhance the accumulation and synthesis of sugars. On the other hand, protein accumulation is also affected by stressed environment. According to Parida *et al*., (2004), increasing salt concentration gradually decreased the total protein contents in leaves of *Bruguiera parviflora*. Rajesh *et al*., (1999) also reported a decrease in total leaf proteins under higher salt treatment in *Ceriops spp*. However, Ashraf & Fatima (1995) found no significant differences of soluble proteins in leaves of salt tolerant and salt sensitive *Carthamus tinctorius*. In current study, total soluble protein contents in leaves, bark and root were decreased in *A. marina* of region I. These findings of total sugars and proteins are in agreement with previous studies.

Table 3. Growth attributes of *A. marina* plants at two different regions.

Region	Plant height (cm) Mean ± SD	Stem diameter (cm) Mean ± S.D	No of shoots per plant (Mean + SD)	No of leaves per plant (Mean + SD)
Region I	40.29 ± 1.5	7.17 ± 0.73	6.43 ± 0.79	20.86 ± 1.77
Region II	47.71 ± 2.2	6.67 ± 0.79	4.14 ± 0.55	22.0 ± 1.53

Table 4. Chlorophyll and carotenoids in *A. marina* Plants during different months of the year.

Season/ Month	Region I (Shah Bander)					Region II (Jamshoro)				
	Chl a (mg/ g)	Chl b (mg/g)	Total Chl (mg/g)	Chl (a/b) ratio	Carotenoids (mg/g)	Chl a (mg/g)	Chl b (mg/g)	Total Chl (mg/g)	Chl (a/b) ratio	Carotenoids (mg/g)
January	2.38±0.1	0.92±0.2	3.3±0.2	2.58	0.58±0.1	2.13±0.12	0.86±0.11	2.99±0.14	2.47	0.42±0.05
April	2.43±0.2	0.82±0.12	3.25±0.09	2.96	0.62±0.2	2.52±0.15	0.98±0.2	3.5±0.12	2.57	0.68±0.1
July	2.51±0.15	1.18±0.2	3.69±0.12	2.12	0.71±0.12	2.41±0.1	1.2±0.15	3.61±0.2	2.0	0.58±0.08
October	2.44±0.2	1.48±0.15	3.92±0.2	1.64	0.68±0.22	2.62±0.09	1.34±0.11	3.96±0.12	1.95	0.71±0.12

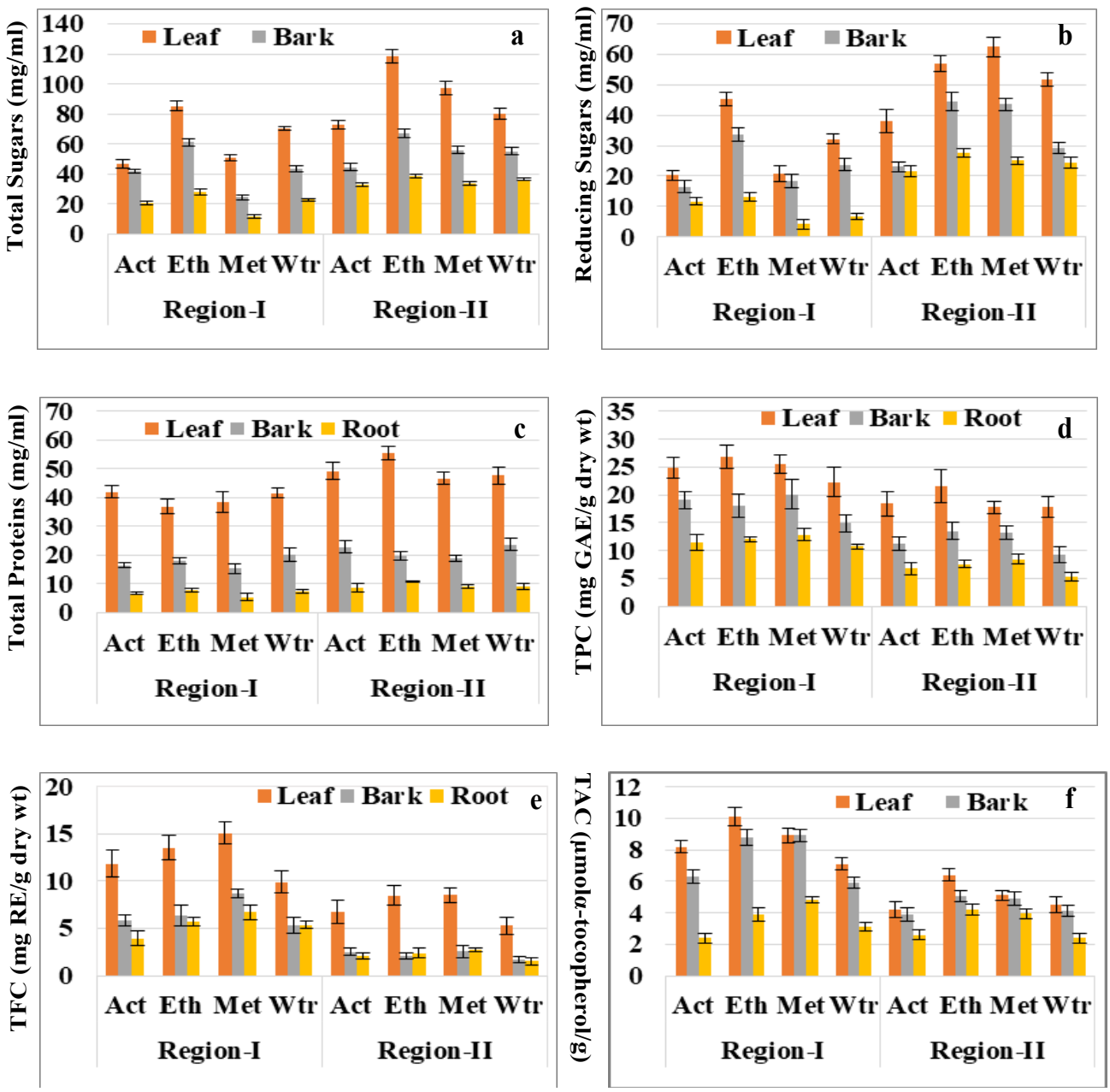

Fig. 2. Phytochemicals findings of various solvent extracts of root, bark and leaf of *A. marina* from region I and II; a) total sugars, b) reducing sugars, c) total proteins, d) total phenolic contents (TPC), e) total flavonoids contents (TFC) and f) total antioxidant capacity (TAC). Act (Acetone), Eth (Ethanol), Met (Methanol), Wtr (Water).

Phytochemical findings: Phytochemical study of various solvents extracts of *A. marina* from both regions revealed the presence of pharmacologically important phenols and flavonoids. The results (Fig. 2d,e and f) revealed that a significantly higher amount of total phenolic contents (TPC), total flavonoids contents (TFC) and antioxidant capacity was found in various extracts of *A. marina* in region I (Shah Bander) then in region II (Jamshoro). According to results, the highest value of total phenolic contents (26.93±2.1 mg GAE/g of dry wt) total flavonoids contents (15.1±0.5 mg RE/g of dry wt) and total antioxidant capacity (10.1±0.55 µmol α tocopherol/g of dry wt) were found in ethanol, methanol and ethanol extracts of leaf of *A. marina* in region I respectively. The presence of phenolic contents in *A. marina* are able to act as primary antioxidants through providing active hydrogen and thus help reducing the damaging effects of oxidative stress. In current study, a significantly highest value of total phenolic acid contents, flavonoids and antioxidants was found in various extracts of *A. marina* of region I as compared to region II (Fig. 2 d, e and f). This may be stressed environment of at Indus delta where plants depend on sea water (salty water) for their growth and development. A lower amount of phenolic acid, flavonoids and antioxidants were observed in *A. marina* of region II. This may be due to the micropropagated plants established in the field were gradually hardened firstly in controlled conditions in the growth room, secondly acclimatized in green house and then finally in field.

In a study Molaeea *et al*., (2017) obtained 18.72±0.14 mg of GAE/g of total phenolic compounds from leaf extracts of *A. marina* but in this study comparatively higher amount of total phenolic contents (26.93±2.1 mg GAE/g of dry wt) was obtained. Furthermore, blowing of strong winds, presences of diverse variety of microorganisms and fauna may also responsible for accumulation and synthesis of these secondary metabolites (Lattanzio, 2003). In current study, the results revealed that different parts of *A. marina* synthesized and accumulated variable amount of secondary compounds. This may be due to external and internal environment of the plant.

The biosynthesis of secondary compounds may vary between different species as well as same species and even different organs of same plant (Del Valle *et al*., 2015). According to Masa *et al*., (2016), organ and season are important factors responsible for the variation in synthesis of secondary compounds in *C. ladanifer*. In current study results revealed that higher amount of phenolic contents, flavonoids and antioxidant were synthesized and accumulated in leaves then other parts of *A. marina* under study. This may be due to synthesis of higher amount of these compounds during growth and development. According to Krischik & Denno (1983), secondary metabolites contents changes during the organ development in plants. Furthermore, abiotic and biotic stresses may change the bioactive compounds as well as site of synthesis which can help to obtain the highest amount of these compounds or suitable time of harvest of raw material of medicinal importance (Lee *et al*., 2005). According to Mittova *et al*., (2004) abiotic stresses such as drought, high salinity, high or low temperatures, high light intensity, mineral deficiencies and herbicide treatment of plants result the disparity between the reduction activity of the antioxidants and the production of reactive oxygen species. In current study accumulation antioxidant was increased in the leaves of *A. marina* of region I (Shah Bander, Indus delta) (Fig. 2f). This increase may be due to salt stress in this region.

Bandeoglu *et al*., (2004) also reported the changes in antioxidant responses in lentil under salt stress. Mittova *et al*. (2003) reported that certain antioxidant enzymes such as catalase, glutathione, reductase and peroxidase are induced in plants which scavenge reactive oxygen species. Alzahrani *et al*., (2019) also reported the increase of ascorbic acid, total antioxidants, catalase (CAT), glutathione reductase (GR), and superoxide dismutase (SOD) in Vicia faba genotypes grown under NaCl stress. These reports also support the current findings of enhanced levels antioxidants produced in the leaves of *A. marina* growing in saline region I (Shah Bander, Indus delta).

Conclusion

Current study provided an efficient method of *In vitro* clonal propagation of *A. marina*. The protocol involve the culturing of nodal stem sections on MS media additionally supplemented with 0.5mg/L BAP, 1.0mg/L Kin and 0.25mg/L of IAA for shoot formation and then transferring microshoots on rooting media containing 1.0mg/L IBA. After gradual hardening, the rooted plantlets were successfully established in new environment in region II. Present findings from different organs of *A. marina* growing in two different environments would help to find the growth pattern of *A. marina* as well as the synthesis and accumulation of phytochemicals in different organs at geographically different area. Through investigating explant source, media components and environment, a more efficient *In vitro* system would be established for higher micropropagation efficiency of *A. marina*. This is the first study in Pakistan in which *A. marina* plants were successfully established in different weather conditions. The study would help in establishing mangrove plants in Jamshoro around the banks of Indus River.

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