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RESEARCH ARTICLE

SALINITY AND PROLINE INDUCED CHANGES IN PROTEIN PROFILE DURING ARTIFICIAL *EX VITRO* MULTIPLE SHOOT INDUCTION IN HYPOCOTYLS OF SOME RHIZOPHORACEAE MANGROVES OF ODISHA

Pramodini Rout and *Uday Chand Basak

Seed Bank and Seed Biology Division, Regional Plant Resource Centre
R & D Institute of Forest and Environment Department, Bhubaneswar-15, Odisha, India

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ABSTRACT

Protein profiling during *ex vitro* multiple shoot regeneration has been reported in three Rhizophoraceae mangroves viz. *Bruguiera parviflora*, *Kandelia candel and Rhizophora apiculata* occurring in Odisha. The effect of exogenous application of NaCl and proline on protein changes was evaluated during the process of multiple shooting in treated and non-treated hypocotyls. The polypeptides of the molecular weight (MW) 29 & 20 KDa appeared to be common in untreated hypocotyls of all the studied species. *R. apiculata* synthesized an unique polypeptide of MW 14 KDa in normal hypocotyls. In *K. candel*, NaCl induced synthesis of 26 & 17 KDa polypeptides. Proline facilitated synthesis of new polypeptides of MW 43 & 32 KDa in *R. apiculata* and 40 & 33 KDa polypeptides in *B. parviflora.* Thus, altered protein profiles be treated as possible markers for evaluating species-specific ability towards *ex vitro* multiple shoot regeneration in Rhizophoraceae mangroves as influenced by salinity and proline.

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INTRODUCTION

The viviparous true mangroves belonging to the family Rhizophoraceae are important intertidal forest communities of tropical and sub tropical region having great ecological and economic significance in providing forestry and fishery products to a large human population, protecting coastal zones from erosion, storms, foods and also in supplying food and shelter for a large no of fishes. However, several anthropological activities have resulted in gradual depletion of this valuable and vulnerable unique resource worldwide in general and Odisha (India) in particular. Mangrove reforestations by seed or propagule planting are common and conventional way to regenerate new individuals especially for tree mangroves. Greater demand and extensive use of propagules of *Rhizophora*, *Kandelia* and *Bruguiera* spp especially for plantation and reforestation is one of the causes of their rapid depletion from primary habitats. *Ex vitro* multiple shoot regeneration technique is now being practiced for clonal multiplication and conservation of valuable species of Rhizophoraceae mangroves. The method of multiple shoot regeneration through decapitation of hypocotyls of *Bruguiera gymnorrhiza* (Basak and Das, 2002) indicates that numerous juvenile shoots evolved from upper region of hypocotyls can give rise to explants potentially suitable for making stem-cuttings/air-layers for vegetative propagation. However, no record is available on bio-molecular basis of *ex vitro* induction of multiple shoots especially in Rhizophoraceae mangroves. Protein synthesis is an essential bio-molecular process during cell division and growth. Salinity impairs normal growth and overall development process in plants. Proline directly or indirectly plays an important role in protein accumulation and in cell adaptation to salinity stress through osmoregulation (Deivanai *et al.*, 2011; Gasper *et al.*, 2002; Demir, 2000). The present study was undertaken to examine species specific alteration of protein profiles influenced by exogenous application of salt (NaCl) and Proline during *ex vitro* multiple shoot regeneration in above three Rhizophoraceae mangroves occurring in Odisha coast of India.

MATERIALS AND METHODS

Viviparous, healthy and mature hypocoytls of Rhizophoraceae mangroves viz. *Bruguiera parviflora*, *Rhizophora apiculata* and *Kandelia candel* were collected from the mangrove forest of Odisha coast (20^0 4'-20^0 8' N and 86^0 45'-86^0 50' E). ***Induction of multiple shoots***: Hypocotyls of all 3 species having uniform length and diameter (species wise) were decapitated in the collar region (meristematic junction between plumule and radical) and grown in polybags containing a mixture of garden soil and sand (1:1) kept under mist system in green house (Basak and Das 2002) (Fig.1).

Corresponding author: Uday Chand Basak,
Seed Bank and Seed Biology Division Regional Plant Resource Centre
R & D Institute of Forest and Environment Department, Bhubaneswar-15, Odisha, India.

Fig.1. Induction of multiple shoots by decapitation of hypocotyls (*Kandelia candel (front), Rhizophora apiculata Bruguiera parviflora (back)*

Treatments

The decapitated hypocotyls were treated every day with 200 mM NaCl (T1), 100 ppm proline (T2) and a combination of NaCl (200 mM) plus proline (100 ppm) i.e T3, maintaining a 'control' where no salt and/or proline treatments were applied. To analyze changes in protein profile (polypeptides) during multiple shoot regeneration, samples were collected at different time intervals i.e at Day-0, Day-7 and Day-15 of decapitation in order to obtain time-bound and species-specific alteration of polypeptides.

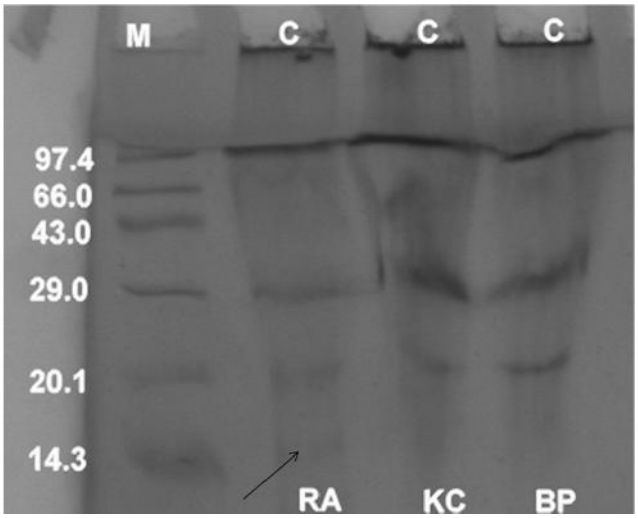

Fig. 2. (Gel-1):SDS-PAGE analysis of protein profile during multiple shooting at Day-0 in Untreated, Control (C) hypocotyls. Band Status: Lane1>MW Marker (M);Lane 2> 29,20 & 14KDa in *Rhizophora apiculata* (RA); Lane 3>. 29&20KDa in *Kandelia candel* (KC) and Lane 4>29&20KDa in *Bruguiera parviflora* (BP).The unique polypeptide having MW 14 KDa was traced out only in RA (Lane 2, arrow marked)

Quantitization of total protein

The fresh decapitated part of the hypocotyls (from all test samples, 500 mg each) was homogenized with pre-chilled mortar and pestle in ice-cold protein extraction buffer (5ml) (pH 7.9). The crude homogenate was centrifuged at 10,000 rpm at 4°C for 30 mins, pellets were washed with 10% TCA and were incubated overnight at 4^0C. Pellets were suspended in 2 ml of 0.1N NaOH. Estimation of total soluble protein was made according to Lowry *et al*., (1951) to facilitate further protein profiling through SDS PAGE.

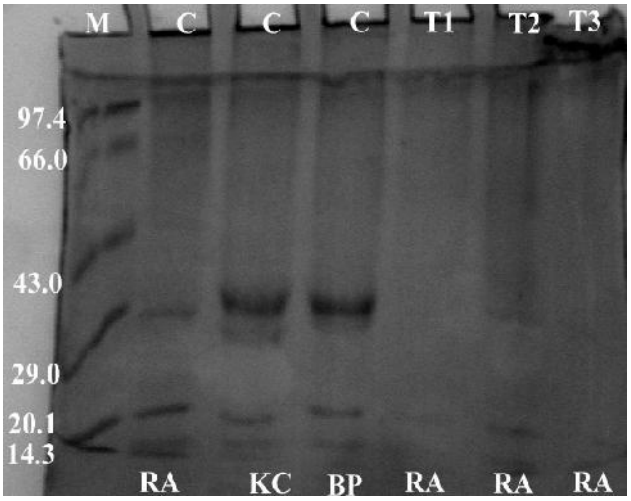

Fig.3. (Gel-2):SDS-PAGE analysis of protein profile during multiple shooting at Day-7 in Untreated, Control(C) hypocotyls. Band Status:Lane1> MW Marker (M); Lane 2-4> showing presence of 29,20 & 14KDA polypeptides in all spp. i.e., *Rhizophora apiculata* (RA), *Kandelia candel* (KC) and *Bruguiera parviflora* (BP);Lane 5> Treated samples of RA produced 20 KDa polypeptide in T1(NaCl 200ppm) sample;Lane 6>presence of 43,32,29, 20 & 14KDa polypeptides in T2(Proline 100ppm);Lane 7>showing 29 & 14KDa polypeptides in T3(NaCl 200ppm + Proline 100ppm).

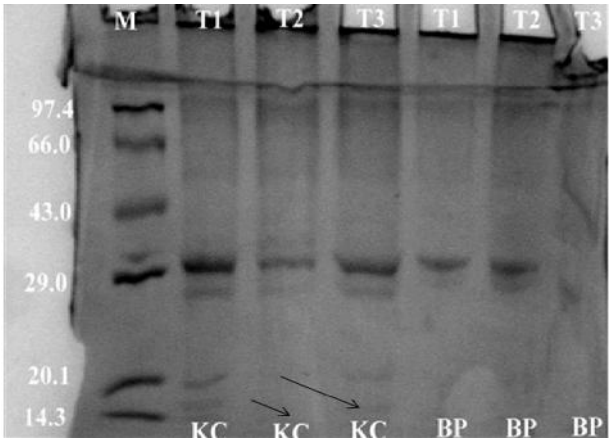

Fig.4 (Gel-3):SDS-PAGE analysis of protein profile during multiple shooting in Treated (Day-7) hypocotyls. Band Status: Lane 1> MW Marker (M); Lane 2&4>*Kandelia candel* (KC) exhibited bands of 29,26,20,17 & 14 KDa polypeptides in T1(NaCl 200ppm) &T3(NaCl 200ppm + Proline 100ppm). Among these, 20 & 14 KDa bands re-appeared due to T3(long arrow);Lane 3>only 29 KDa band found in T2(Proline 100ppm)., the other bands of 20 & 14 KDa disappeared(short arrow) Lane 5&6> *Bruguiera parviflora* (BP) showed presence of 29 & 26 KDa polypeptides; Lane 7>bands appeared in T1&T2 found missing in T3 except a faint band of 14 KDa. in the sample of *B. parviflora*

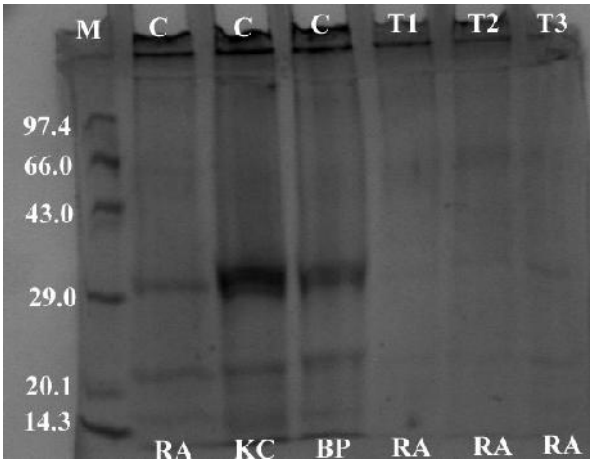

Fig.5. (Gel-4): SDS-PAGE analysis of protein profile during multiple shooting in Treated (Day-15) hypocotyls. Band Status: Lane1> MW Marker (M); Lane 2-4> showing presence of 29,20 & 14KDA polypeptides in all spp. i.e., Rhizophora apiculata (RA), Kandelia candel (KC) and Bruguiera parviflora (BP);Lane 5-6> in RA, T1 & T2 did not produce any polypeptides. Lane 7> Low intensity bands of MW 29,20 & 14 KDa were visible due to T3 in RA.

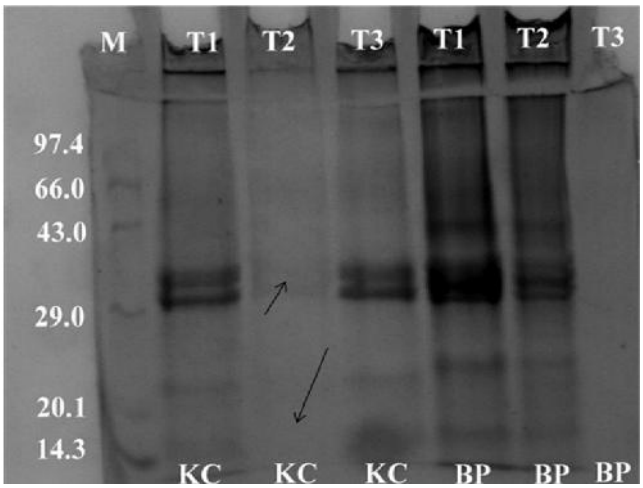

Fig.6. (Gel-5): SDS-PAGE analysis of protein profile during multiple shooting in Treated (Day-15) hypocotyls. Band Status: Lane 1> MW Marker (M); Lane 2&4>*Kandelia candel* (KC) exhibited bands of 29,26,20 & 14 KDa polypeptides in T1(NaCl 200ppm) &T3(NaCl 200ppm + Proline 100ppm). Lane 3>bands of 26,20 and 14 KDa found in T1 were disappeared (long arrow) in T2 except 29 KDa (short arrow);Lane 5&6> *Bruguiera parviflora* (BP) showed presence of 40,33,29,20 & 14 KDa polypeptides both in T1 & T2; Lane 7>bands appeared in T1&T2 found missing

Analysis of protein profile through SDS-PAGE during multiple shoot regeneration

The protein profiles were recorded through SDS-PAGE following standard method. The protein molecular weight marker (PMWM, range 14.3 KDa to 97.4 KDa) from Bangalore Genei, India was used as standard. The supernatant sample containing 40 µg (aprox.) of protein were mixed with equal volume of solubilizing buffer containing 300 mM Tris-HCl (pH 6.8), 60% glycerol, 12% SDS, 864 mM 2-mercaptoethanol and 0.05% bromophenol blue and heated for 5 min at 90-95^{0}C. Gel was made according to Laemmli (1970). A 11% separating gel containing 37.5% of 1M Tris (pH 8.8), 27.4% of 40% acrylamide, 0.025% SDS, 0.04% ammonium persulfate and 0.23% TEMED was used for resolving whereas a 4.4% stacking gel containing 3.3% of 1M Tris (pH 6.8), 6% of acrylamide, 0.022% of SDS, 0.09% of APS and 0.05% TEMED was used to concentrate the polypeptides. The electrophoresis running buffer consisted of Tris (0.3%) pH 8.3, glycine (1.44%), SDS (0.2%). Electrophoresis was conducted at 60 mA for 4h using a Genei make Gel Electrophoresis system. The gels were stained with 0.25% Coomassie Brilliant blue R-250 (Sigma) in 50% (v/v) methanol and 10% acetic acid for overnight. The gels were then distained (5% methanol & 10% acetic acid) to make gel bands prominent by clearing excess stain on the gel. The gels were photographed using Gel-Doc System GS-710. The protein profile was analysed using the compatible software (Launch Genei).

RESULTS

Changes in protein profile during multiple shoot regeneration through SDS-PAGE

SDS-PAGE analysis of protein profiling in control (untreated hypocotyls) of *Kandelia candel*, *Rhizophora apiculata* and *Bruguiera parviflora* (Fig.2, Lane 2-4) indicated that polypeptides having molecular weights 29 KDa, 20 KDa were appeared in all species as common bands. However, in case of *R. apiculata*, an unique polypeptide having MW 14 KDa was traced out in un-treated Day-0 control sample (Fig.2, Lane 2, arrow). Moreover, the polypeptide of 14 KDa was also appeared in untreated decapitated hypocotyls of Day-7 and Day-15 samples of *B. parviflora* and *K. candel* along with *R. apiculata* (Fig.3, Lane 2, 3, 4; Fig.5, Lane 2, 3, 4) In *K. candel*, regeneration of multiple shoot witnessed synthesis of 5 polypeptides having molecular weights 29, 26, 20, 17 and 14 KDa at different stages of induction affected by salinity stress and proline application over a period of 15 days (Fig.2, Lane 3; Fig.3, Lane 3; Fig.4, Lane 2,3,4; Fig.5, Lane 3; Fig.6, Lane 2,3,4). The salinity induced protein synthesis during multiple shooting was evident at Day-7 by presence of polypeptide bands of molecular weights 26 and 17 KDa (Fig.3, Lane 3); these polypeptides were not existed in normal (Day-0, control) hypocotyls (Fig.2, Lane 3). At Day-15, the bands of 29, 26, 20 & 14 KDa polypeptides were visible (Fig.6, Lane 2). Proline application alone, however, did not show presence of any new polypeptide at Day-7. Moreover, the 20 KDa polypeptide of control sample was disappeared leaving a single band of 29 KDa polypeptide (Fig.4, Lane 3, short arrow). At Day-15, bands of 26, 20 & 14 KDa were disappeared (Fig.6, Lane 3, long arrow) while a band of 29 KDa reappeared (Fig.6, Lane 3, short arrow). In 'NaCl plus proline' treated samples, the counter effect of proline over NaCl was evident by disappearance of 26 and 17 KDa polypeptides at Day-7 of multiple shooting (Fig.4, Lane 4, long arrow). At Day-15, no band was found. In *R. apiculata*, regeneration of multiple shoot witnessed synthesis of 5 polypeptides having molecular weights 43, 32, 29, 20 and 14 KDa at different stages of induction affected by NaCl stress and proline application (Fig.2, Lane 2; Fig.3, Lane 2,5-7; Fig.5, Lane 2,5-7). The NaCl

induced protein synthesis was impaired drastically during multiple shooting at Day-7 which was evident by early disappearance of two polypeptides of 29 and 14 KDa followed by further disappearance of 20 KDa polypeptide (Fig.3, Lane 5). All these polypeptides were very much present in normal (control) hypocotyls. At Day-15, no polypeptide band was found (Fig.5, Lane 5). Proline application induced synthesis of two new polypeptides having molecular weights 43 and 32 KDa at Day-7 of regeneration of multiple shoots (Fig.3, Lane 6). At Day-15, all the above mentioned polypeptides were found disappeared (Fig.5, Lane 6). In 'NaCl plus proline' treated samples, the counter effect of proline over NaCl was evident by reappearance of all polypeptides with 29, 20 and 14 KDa which were found present in normal (control) hypocotyls (Fig.3, Lane 7). At Day-15, low intensity bands of molecular weights 29, 20 and 14 KDa were visible (Fig.5, Lane 7).

In *B. parviflora*, regeneration of multiple shoot triggered synthesis of 6 polypeptides having molecular weights 40,33,29, 26, 20 and 14 KDa at different stages of induction affected by salinity stress and proline application (Fig.2, Lane 4; Fig.3, Lane4; Fig.4, Lane 5-7; Fig. 5, Lane 4; Fig. 6, Lane 5-7). At Day-7, the salinity induced protein synthesis during multiple shooting was evident by presence of polypeptide bands of molecular weights 40, 33, 26 and 14 KDa (Fig.4, Lane 5); these polypeptides were not found in normal (control) hypocotyls (Fig.4, Lane 5). At Day-15, polypeptides of 40, 33, 29, 20 & 14 KDa were found (Fig.6,Lane 5). Proline application alone, however, did not show presence of any new polypeptide. Moreover, the 40 and 33 KDa polypeptides were found disappeared (Fig.4, Lane 6). At Day-15, polypeptides having molecular weights 40,33,29,20 and 14 KDa were synthesized (Fig.6, Lane 6). In 'NaCl plus proline' treated samples; the counter effect of proline over NaCl was evident by disappearance of all polypeptides except 14 KDa which was appeared at Day-7 of multiple shoot regeneration (Fig.4, Lane 7). At Day-15, no band could be traced out (Fig.6, Lane 7).

DISCUSSION

Efficient *ex vitro* shoot regeneration in hypocotyls of mangrove Rhizophoraceae i.e *K. candel, B. parviflora*, and *R. apiculata* were affected by exogenous application of sodium chloride (NaCl) and proline. In normal (control) conditions, two polypeptides having molecular weight of 29, 20 KDa were found 'common' in all the three studied species. One unique polypeptide of 14 KDa was, however, identified in *R. apiculata* which may act as indicator of high salt stress conditions. Apart from 29, 20 and 14 KDa polypeptides, synthesis of various other high molecular weight polypeptides occurred due to exogenous application of NaCl and proline. Multiple shoot regeneration much affected by exogenous application of NaCl and proline in *K. candel* and *B. parviflora*, the less salt tolerant intertidal species. In *K. candel*, additional polypeptide of 26 and 17 KDa were appeared at Day-7 due to NaCl treatment. Where as, *B. parviflora*, synthesized 40, 33, 26 and 14 KDa polypeptides at Day-7 of decapitation. In a previous study, it was reported in *B. parviflora* that the salt stress affected synthesis of 90, 50, 33, 23 and 16 KDa polypeptides (Dasgupta *et al*., 2012; Parida *et al.*, 2004). *R. apiculata* did not respond exogenous application of NaCl; polypeptides of 29, 20, and 14 KDa were missing during the course of multiple shoot regeneration. In this study, proline application counteracted the effect of NaCl-salinity which was evident by disappearance of specific polypeptides which were apparent during NaCl treatment. Proline treatment facilitates synthesis of new polypeptides 43, 33 KDa in *R. apiculata*. The effect of proline was not prominent in other two species where no new polypeptide was synthesized. Proline also induced synthesis of 40 & 33 KDa polypeptides in *B. parviflora*. In 'NaCl plus proline' treated hypocotyls, the counteract of proline over NaCl-salinity was evident by disappearance of all most all polypeptides which were appeared due to NaCl treatment. Proline directly or indirectly plays an important role in protein accumulation and in cell adaptation to salinity stress and enhance available source of energy and reducing power (Muayed *et al*., 2012; Okuma *et al*., 2004)

Conclusion

Since multiple shoot regeneration (*ex vitro*) in hypocotyls of Rhizophoraceae mangroves are interlinked with synthesis of various polypeptides due to influence of salt and proline, it may be concluded that further studies on genetic regulation on the above scores may open new avenue for evaluating species-specific respond pertaining to multiple shoot regeneration.

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