International Journal of Biodiversity and Conservation Vol. 2(7) pp. 162-172, July 2010
Available online http://www.academicjournals.org/ijbc
ISSN 2141-243X ©2010 Academic Journal

Full Length Research Paper

Conservation of *Aegiceras corniculatum* (L.) Blanco (River mangrove, Khalsi): A new approach of vegetative propagation through hypocotylar juvenile stem cuttings

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Accepted 19 March, 2010

Clonal propagation method through induction of adventitious rooting in hypocotyl-plus-stem (HpS) and juvenile-stem (JS) cuttings was reported in *Aegiceras corniculatum* (L.) Blanco (khalsi), mangrove plant of coastal Orissa, India. The induction of adventitious roots is an essential process in vegetative propagation. Adventitious rooting in above cuttings was induced by exogenous application of root promoting substances (RPS) viz. IBA and NAA in four combinations under mist house conditions. Significant increase in rooting response (76.70%) and root number (4.48 per cutting) were recorded in the HpS cuttings treated with IBA 1.0 mg/l + NAA 5.0 mg/l (T2). However, the JS cuttings under same treatment (T2) showed maximum root length (4.30 cm per cutting). Though, untreated HpS cuttings responded to induction of rooting (16.70 %), JS cuttings failed to produce any adventitious root without RPS. Anatomically, all the treated cuttings responded to rooting process by forming 'root primordia' after 10 days of treatment in HpS and 20 days in case of JS cuttings. The 'root emergence' took place after 30 days of treatment in HpS and 40 days in case of JS cuttings. Biochemically, prompt and significantly highest adventitious rooting capability (in terms of percent rooting and mean root number per cutting) of HpS cuttings might be due to presence of higher level of indigenous storage carbohydrate (starch and soluble sugar) and soluble protein in the rooting zone as compared to JS stem cuttings. The present study, thus, highlights a viable process of adventitious root formation by analyzing anatomical and biochemical evidences which may open a new avenue for mass production of planting materials through clonal propagation using cryptoviviparous hypocotyls of *Aegiceras corniculatum*, a naturally depleted but economically important mangrove plant of Orissa, India.

Key words: Adventitious rooting, carbohydrate, protein, root primordia, root promoting substances.

INTRODUCTION

Aegiceras corniculatum (L.) Blanco is a small evergreen tree mangrove belonging to the family Myrsinaceae which exhibits crypto-viviparous germination through production of curved green radicle (called hypocotyls) when fell down and reaching soil substratum; the radicle eventually penetrates soft soil substratum protruding out the plumule upwardly to form apical leafy shoot. Globally, however, natural regeneration of this species reported to become highly restricted due to excessive and uncontrolled browsing of young seedlings by deer (Siddiqi and Husain, 1994), predation of hypocotyls by crabs (Robertson et al., 1994; Farnswarth and Ellison, 1991) and the state of Orissa is not exceptional too. Though, population of mature plants of *A. corniculatum* is sporadically existing in Bhitarkanika mangrove forests of Orissa, young and juvenile plantlets are rarely visible (Upadhya and Mishra, 2008) Moreover, tree mortality happened to be in alarming rate due to erosion of riverbank/estuary, wind/storm injury and pest infestation (Upadhya and Mishra, 2008; Goh and Yipp, 1996; Seeman and Walter, 1995).

In order to compensate such multifarious constrains of

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Abbreviations: HpS, Hypocotyl-plus-stem; **JS,** juvenile-stem; **RPS,** root promoting substances; **T1,** treatment-1 (IBA1.0 mg/l + NAA2.0 mg/l); **T2,** treatment-2 (IBA1.0 mg/l + NAA5.0 mg/l); **T3,** treatment-3 (IBA3.0 mg/l + NAA2.0 mg/l); **T4,** treatment-4 (IBA3.0 mg/l +NAA5.0 mg/l); **T0,** treatment-0 (control, without RPS).

natural regeneration, application of vegetative propagation method would be one of the right options for rejuvenating balanced population of *A. corniculatum* in the mangrove ecosystems. There are, however, few reports on vegetative propagation of this species through exogenous application of root promoting substances (RPS) in mature/semi-mature stem cuttings and air-layers which may cause further depletion of mature and reproducing trees in the wild (Basak et al., 2003; Eganathan et al., 2004). Pressure on adult plants can be avoided by utilizing mature hypocotyls (to be collected before predations) as a source of explants for vegetative propagation with the aid of promising combinations of RPS viz. IBA and NAA based on earlier available report (Basak et al., 2003). The biochemical and anatomical events leading to adventitious rooting in air-layers were reported on the basis of enzyme activities and C:N metabolism (Basak et al., 2003). Since, carbohydrate and protein provide vital sources of biochemical energy, present study has given emphasis on analysis of those biomolecules during formation of adventitious roots in *A. corniculatum.* In this context, an attempt has been made for clonal propagation by inducing artificial rooting in hypocotylar-plus-stem (HpS) cuttings (a 2-3-noded newly developed shoot having a portion of hypocotyls at the base of the cuttings) and juvenile-stem (JS) cuttings (a 2-3 noded newly developed shoot devoid of hypocotylar part) along with their bio-anatomical interpretations to mitigate the prevailing problem of lack of natural young seedlings of *A. corniculatum* for conservation through production of planting materials round the year.

MATERIALS AND METHODS

Plant materials

Mature and healthy hypocotyls were collected from adult and reproducing trees in Bhitarkanika mangrove forest situated at 20°40' - 20°80' N Latitude and 86°45' - 87°50' E Longitude, in the north-eastern coastal Orissa, India. The hypocotyls consisted of pedicellate fruit which dehisces early to expose green radicle curving away from the fruit wall.

Rearing and preparation of cuttings

Hypocotyls were grown in polybags containing garden soil and sand mixture and kept under mist systems for germination to produce explants suitable for preparation of cuttings. The explants were selected for rooting treatment when juvenile shoots became 6 - 8 cm long having 2 - 3 leafy-nodes (tender shoots) and whole collections were divided into two groups. While one group was meant for preparing cutting of juvenile shoots along with a basal portion of hypocotyls (HpS), the other group was kept for preparation of juvenile shoot cuttings without hypocotylar part (JS).

Treatments

Cuttings (HpS and JS) made from both the explant-groups, were treated with root promoting substances (RPS) at four combinations (T1-T4) that is, IBA1.0 + NAA2.0 (T1), IBA1.0 + NAA5.0 (T2), IBA3.0 + NAA2.0 (T3) and IBA3.0 mg/l +NAA5.0 mg/l (T4) with a control (T0) where no RPS was applied. The above range of concentrations and combinations of RPS has been selected on the basis of their efficiency recorded earlier during rooting in air-layers of *A. corniculatum* (Basak et al., 2003).

Anatomical studies

The basal portion (2 - 3 cm, called rooting zone) of both treated and untreated (control) cuttings were sampled at 0, 5, 10, 15, 20, 25, 30 and 40th day to record differential stages of root development, that is, from initiation to emergence of adventitious root formation. Samples were fixed in formaldehyde-acetic acid-ethanol solution (FAA, 1:2:17) and subsequently preserved in 70% ethanol. Uniform and thin transverse sections (40 - 50 µm) of the rooting zone were cut in sledge microtome (Spencer make, model 1010-SMT-009). Selected sections were stained in saffranin followed by fast green (double staining) following a series of alcohol dehydration and mounted in DPX (Dwivedi and Singh, 1985). The sections were examined under compound microscope to record anatomical changes during initiation and formation of adventitious root and documented through photomicrography (Nikon Microscope, Eclipse 50i, Japan).

Biochemical studies

The basal rooting zone (2 - 3 cm) of the treated and untreated cuttings were sampled at 0, 5, 10, 15, 20, 25, 30 and 40th day of treatment for biochemical analysis.

Estimation of carbohydrates

Total carbohydrate (soluble sugar and starch) was estimated using phenols-sulphuric acid method (Dubois et al., 1956) with some modifications.

Total soluble sugars

The fresh test sample (500 mg each) was grounded in a mortar and pestle with 10 - 15 ml of ethanol and left overnight and homogenates were centrifuged at 5000 rpm for 15 min, the supernatants were collected and concentrated on a water-bath. These aqueous concentrates were used for soluble sugars after making necessary dilution with distilled water. The residual pellets obtained by centrifugation were used for starch estimation.

Starch

The pellet-residues of each test sample was suspended in a mixture of 5 ml of perchloric acid (50%) and 5 ml of distilled water, centrifuged at 5000 rpm for 15 min. The supernatants were collected following 2 - 3 times repetition of the above steps. The volume of pooled supernatant of each sample was made up to 50 ml with distilled water and used for estimation of starch.

Quantitisation of carbohydrates

Aliquots (1 ml) of each of the test sample obtained for soluble sugar and starch were used to quantifying the total carbohydrates. To each of these, 1 ml of 5% aqueous phenol was added quickly and shaken gently keeping the test tubes on ice-block for 5 min. Then

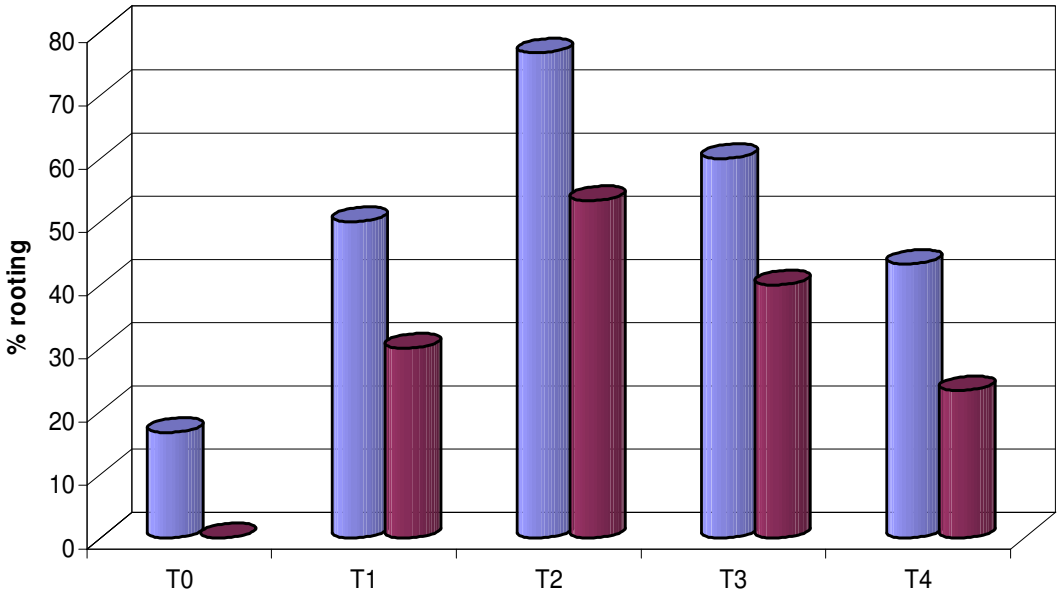

Figure 1. Rooting percentage recorded in un-treated (T0) and treated (T1 - T4) cuttings.

5 ml of Conc. H_2SO_4 was added gently; the tubes were kept under tap water (30 ± 5°C) for 15 min and the optical density (OD) of the orange-yellow colours thus developed were taken at 490 nm in spectrophotometer (Bioline Specord50, Analytik Jena make). The concentration (mg/g fwt) of the total soluble sugars was directly calculated from the regression curve using glucose as standard. The sugar content in terms of glucose equivalent and the use of conversion factor (0.9 to convert the values of glucose to starch (mg/g fwt)) was made in each sample.

Protein estimation

Extraction: Fresh sample (1 g from rooting zone) was homogenized in 10 ml of cold TCA (10%) for 30 min; kept at 4°C overnight and then centrifuged (Osborne, 1962). The pellet was suspended again in 5% TCA (5 ml) and heated on a water bath at 80 ± 5°C for 20 min. After cooling, the suspension was re-centrifuged and the pellet so obtained was washed with distilled water. Finally, after centrifugation of the aqueous suspension, the pellet-residue was dissolved in 1N NaOH (5 ml) and kept overnight at room temperature for quantitative analysis for total protein.

Quantification: The total protein content was estimated using the method of Lowry et al. (1951). To 1 ml of each test sample, 4 ml of freshly prepared alkaline solution (prepared by mixing 50 ml of 2% Na_2CO_3 in 0.1 N NaOH and 1 ml of 0.5%$CuSO_4.5H_2O$ in 1% sodium potassium tartrate) was added at room temperature and kept undisturbed for 10 min. Subsequently, to each of these mixture, tubes 0.5 ml of Folin-Ciocaltcau reagent was added and after half an hour, the OD of each was measured at 750 nm using spectrophotometer against the blank (without protein sample). The total protein content in each sample was calculated by referring the ODs of test sample with the standard curve of BSA.

Statistical analysis

Statistical analysis (tow-way ANOVA) was conducted using Graphpad Prism 5 Software. To analyse the effect of the parameters viz. auxin treatments (RPS T1-T4) and cutting types (HpS and JS), the mean values of each replication were estimated. Each treatment (including control, T0) was replicated thrice with 30 cuttings per replications.

RESULTS

Rooting response

The auxinic compounds IBA and NAA had profound root inducing ability. Both the cuttings (HpS and JS) of *A. corniculatum* exhibited significant variations (at the $p < 0.001$ level) in their rooting ability at different concentrations and combinations. Maximum rooting response (76.70%) and root number (4.48 nos. per cutting) were recorded in the HpS cuttings treated with T2 (Figures 1, 2, 3, 4 and 5). However, the JS cuttings under same (T2) treatment showed significant increase in the root length (4.30 cm per cutting) at the $p < 0.001$ level (Figure 6). Though, untreated HpS cuttings (T0) responded to induction of rooting (16.70%), JS cuttings failed to produce any adventitious root without root promoting substances (Figure 1).

Anatomical studies

By and large, all the treated cuttings responded to rooting process by forming 'root primordia' originated from phloem parenchymatous cells after 10 days of treatment in HpS (Figure 7) and 20 days in case of JS cuttings (Figure 8). Root initials were found to develop as a group of dividing phloem parenchymatous cells and formation of root primordial was evident by presence of

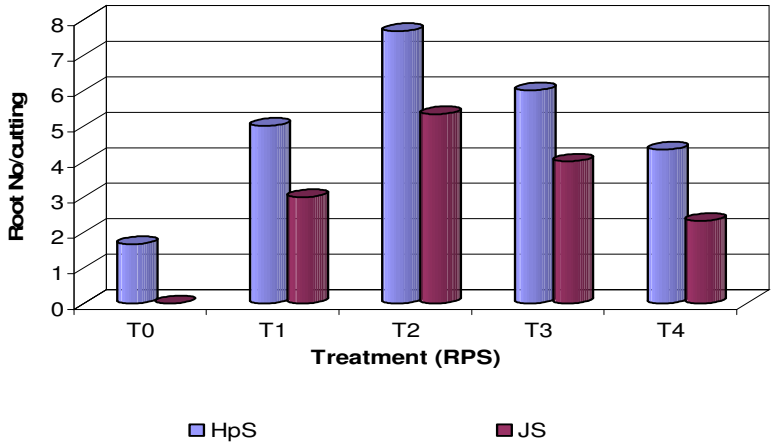

Figure 2. Mean root number per un-treated (T0) and treated (T1 - T4) cuttings.

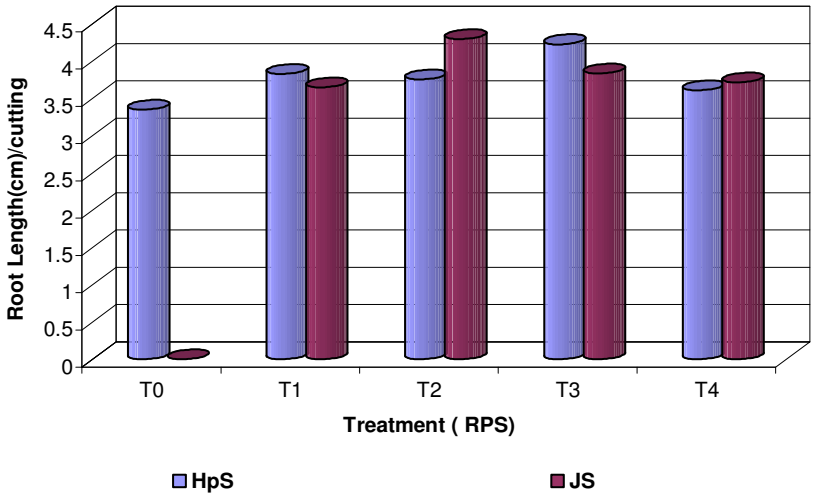

Figure 3. Mean root length (cm) per un-treated (T0) and treated (T1 - T4) cuttings.

Figure 4. Rooting in HpS cuttings (red bar = hypocotyls part, yellow bar = stem) treated with T2.

Figure 5. Rooting in JS cuttings (without hypocotyl part) treated with T2.

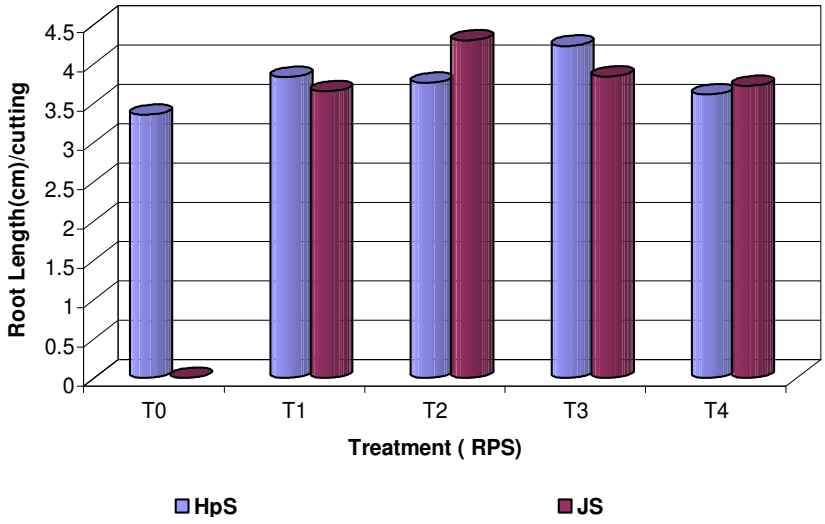

Figure 6. Mean root length (cm) per un-treated (T0) and treated (T1-T4) cuttings.

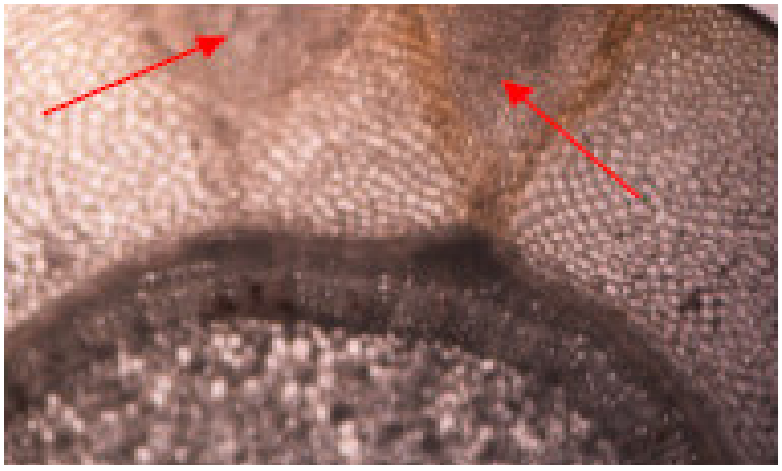

Figure 7. Root primordial initials (arrow) in HpS cutting (T.S.at 10 day).

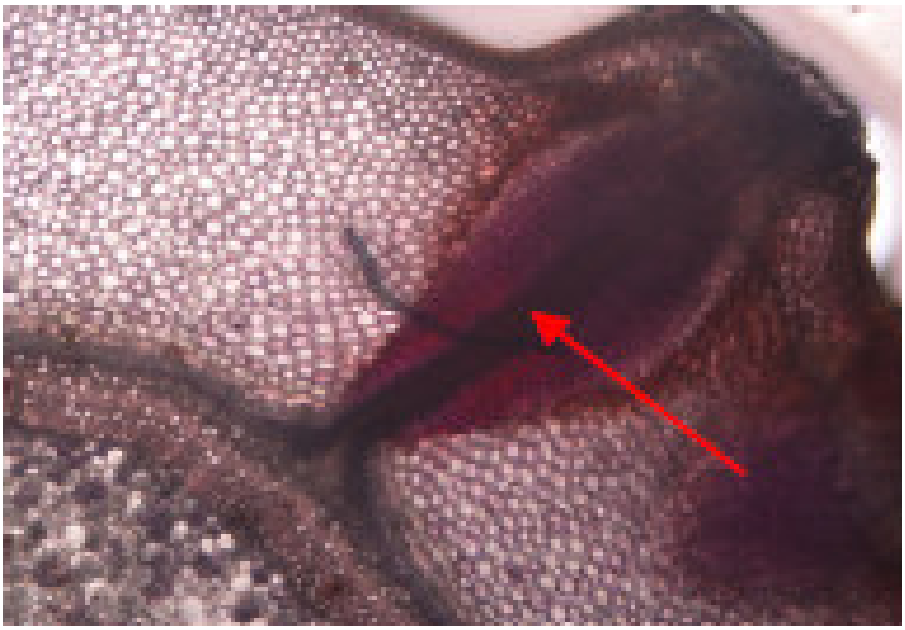

Figure 8. Root primordial in HpS cutting (T.S. with vascular connection, arrow).

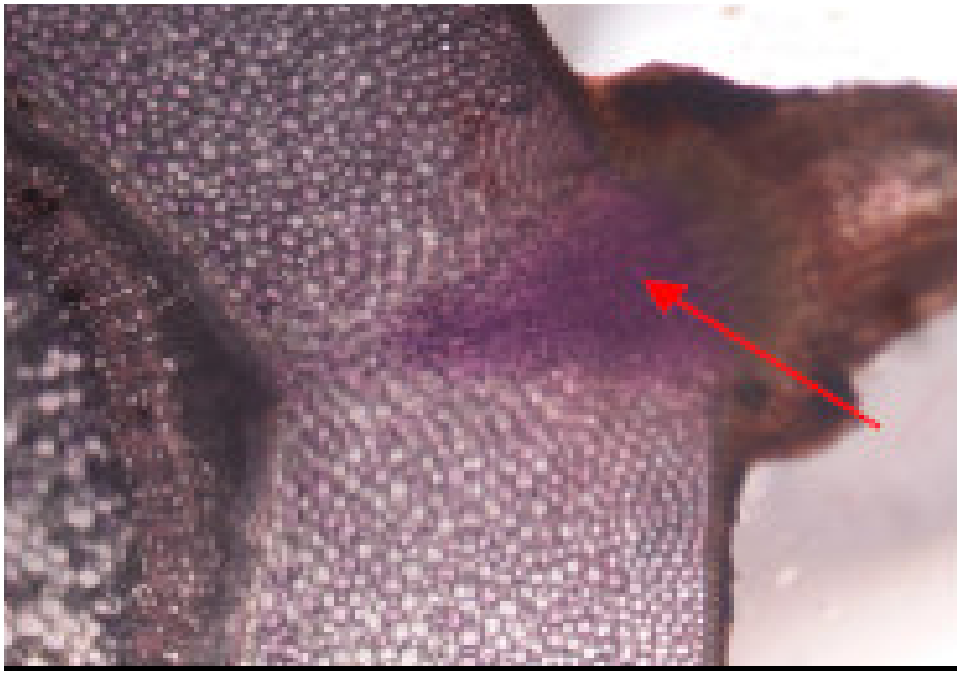

Figure 9. Root primordia initials (arrow) in JS cutting.

'linear-club-shaped' structure in the outer cortical region of JS cuttings (Figure 9) and 'wider-club-shaped' structure in the inner stelar region of HpS cuttings (Figure 10). The 'root emergence' took place after 30 days of treatment in HpS cuttings (Figure 11) and 40 days in case of JS cuttings (Figure 12).

Biochemical studies

Protein

Initial protein content (day 0) in the control cuttings of HpS (13.30 mg/g fwt) was found to be 45.2% higher than

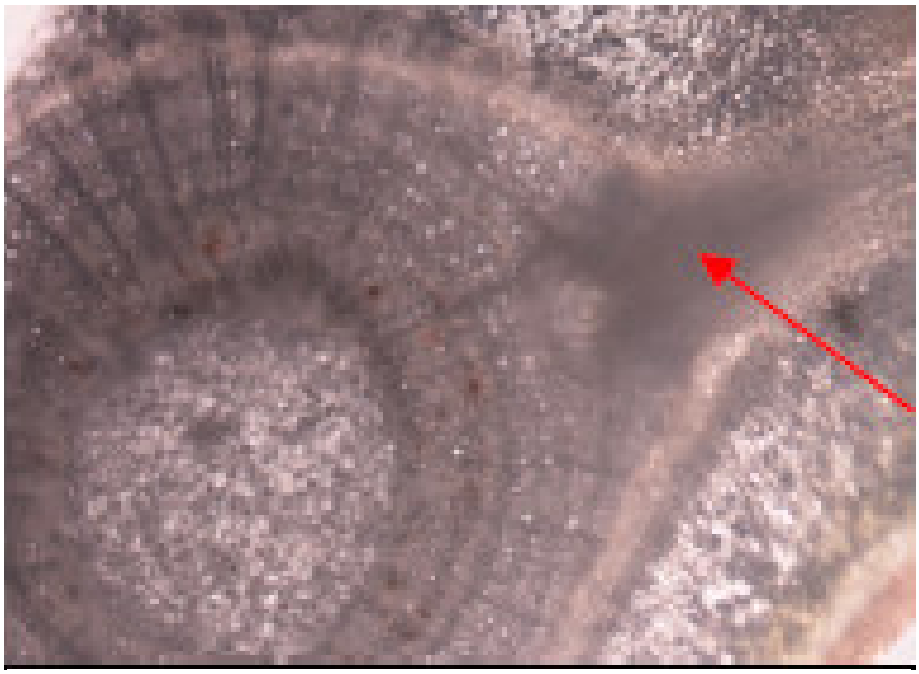

Figure 10. Root primordia (arrow) in JS cutting (T.S. at 20 d).

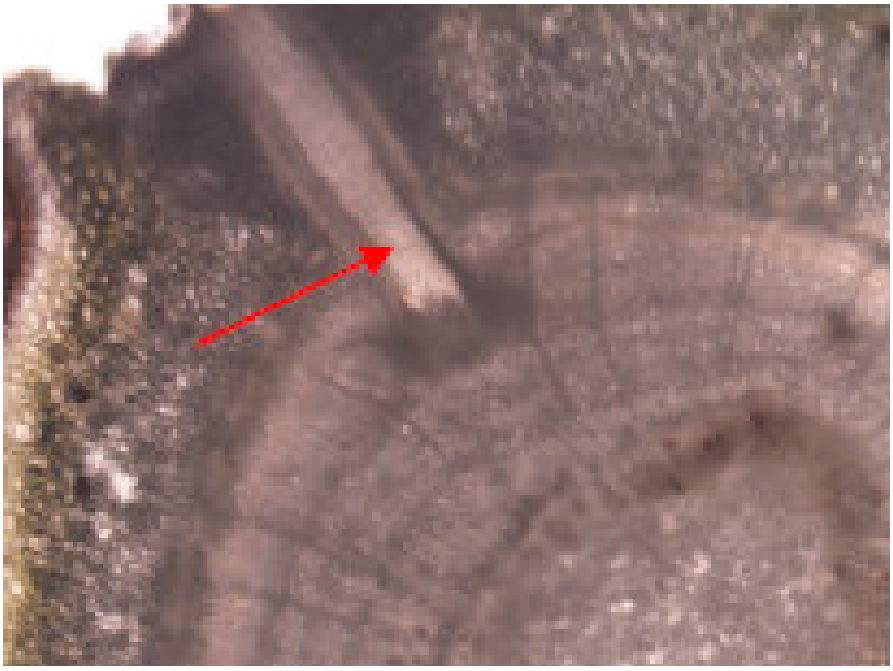

Figure 11. Developed Root primordia in JS cutting (vascular connection, arrow).

that of JS cuttings (9.16 mg/g fwt). Significant variation in protein content was found in HpS cuttings treated with T2 at the $p < 0.001$ level at the day 10 (16.90 mg/g fwt) against the control (13.84 mg/g fwt) followed by JS cuttings with same treatment but at the day 15 (14.66 mg/g fwt) (Figure 13) against control (9.42 mg/g fwt). Exogenous application of RPS viz. IBA and NAA caused an increase in the protein content from the day 0 to the day 10 in HpS cuttings and up to the day 15 in JS cuttings but decreased there after in both cases.

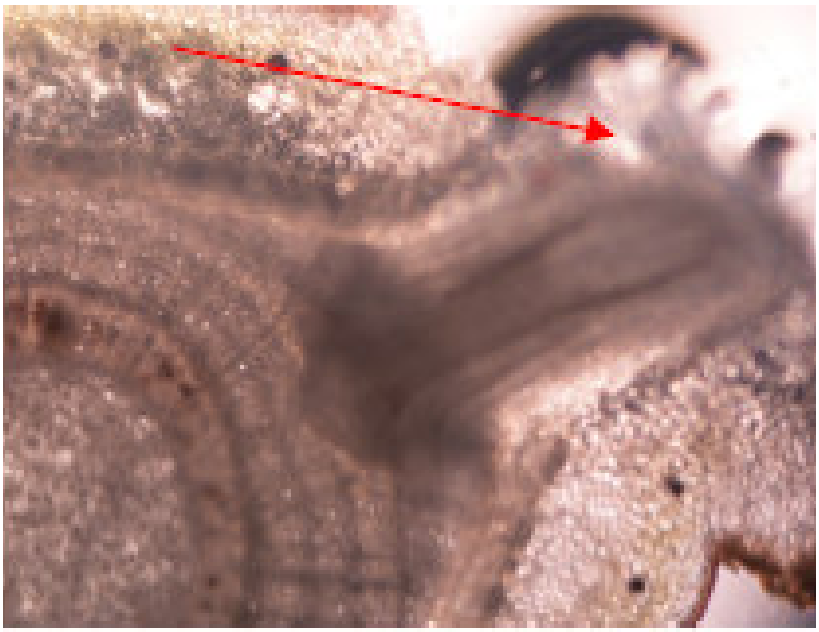

Figure 12. Root emergence (arrow) in JS cutting (T.S. at 40 d).

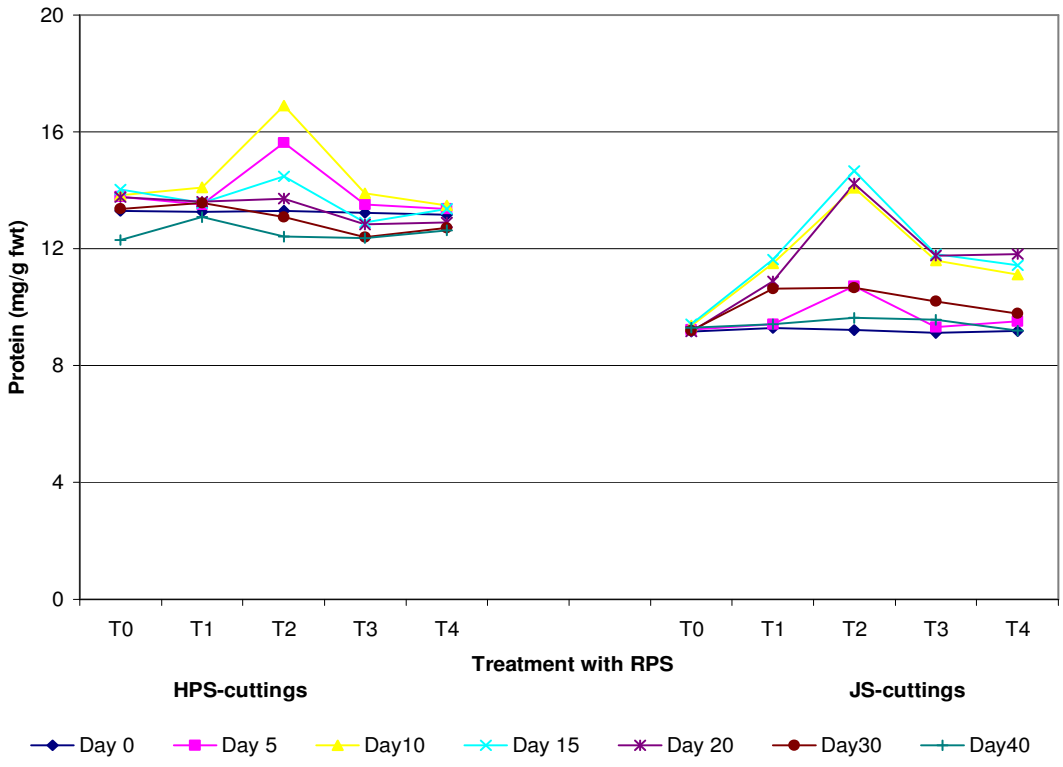

Figure 13. Protein changes during rooting period in HpS (left) and JS-cuttings (right) in response to RPS.

Starch

Starch content (30.43 mg/g), at the day 0, in HpS control cuttings was found to be 44.5% higher than that of JS cuttings (21.06 mg/g fwt). Significant increase (at the level $p < 0.001$) in starch content was found up to day 10

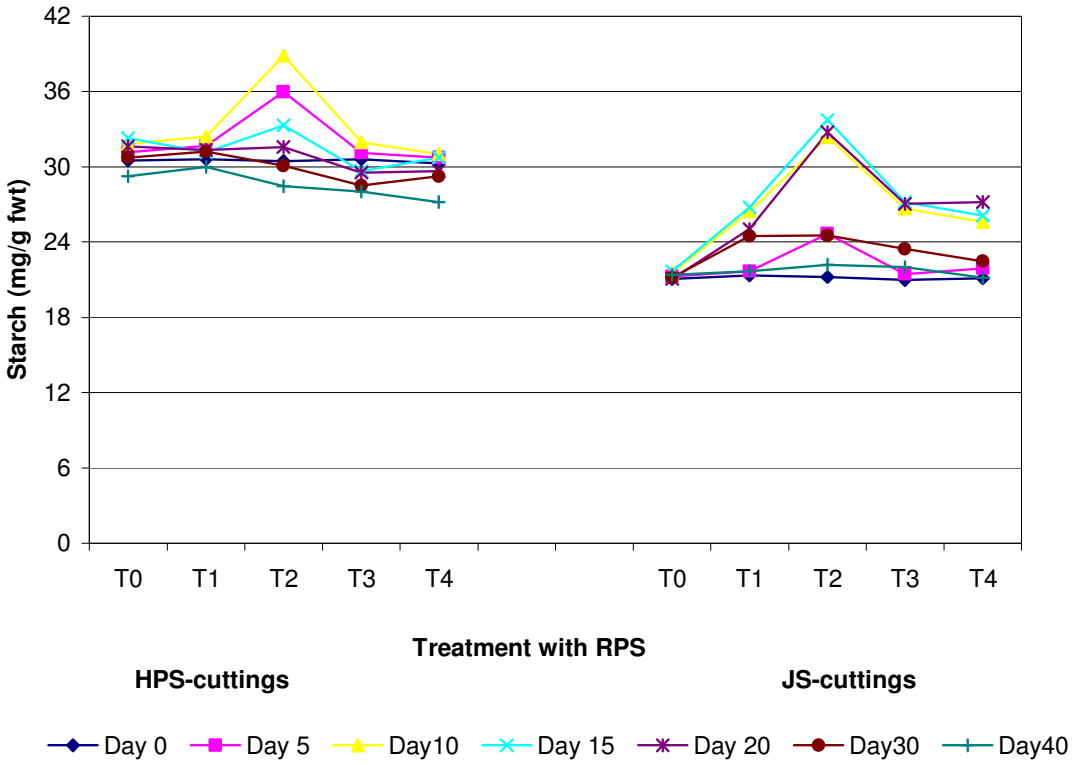

Figure 14. Starch changes during rooting period in HpS (left) and JS-cuttings (right) in response to RPS.

in HpS cuttings with maximum amount of 38.88 mg/g (fwt) in the T2-treated cuttings against the control (31.82 mg/g fwt) followed by gradual decrease in the subsequent period of sampling. In JS cuttings, highest starch content (33.72 mg/g fwt) was obtained at day 15 when applied with T2 as compared to the control (21.67 mg/g fwt). Irrespective of treatments, the increasing trend of starch content was noticed up to day 10 and 15 in HpS and JS cuttings, respectively, followed by gradual decrease during subsequent sampling periods (Figure 14).

Sugar

Total soluble sugar content in the control cuttings of Hps (50.33 mg/g fwt) at day 0, was found to be 44.9% higher than in the JS cuttings (34.72 mg/g fwt). The maximum sugar content was found in HpS cuttings treated with T2 at day 10 (64.12 mg/g fwt) against the control (52.47 mg/g fwt) followed by JS cuttings with same treatment but at day 15 (54.99 mg/g fwt) (Figure 15) against control (35.75 mg/g fwt). All such variations were significant at the $p < 0.001$ level.

Application of RPS viz. IBA and NAA caused an increase in the sugar content from day 0 to day 10 in HpS cuttings and up to day 15 in JS cuttings but decreased there after in both cases.

DISCUSSION

Adventitious root formation capability of *A. corniculatum* varied among the hypocotylar stem cuttings (HpS) and juvenile stem cuttings (JS). Rooting response in terms of percent rooting, root number and rooting time were found better in HpS cuttings as compared to JS cuttings. Increase in the rooting potential of stem cuttings due to initial presence of relatively higher amount of carbohydrate has also been reported (Basak et al., 1995, 2000).

Combined auxin treatment was reported to have strong effect on the rooting response of stem cuttings/air-layers of difficult-to-root species (Basak et al., 1995; Jackson 1986). In the present study, combined treatment with IBA and NAA also produced highest rooting percentage, root number and length in both the types of cuttings over the controls. The stimulatory effects of auxins on adventitious rooting of stem cuttings and air-layers of several other mangrove and non-mangrove species have been reported earlier (Basak et al., 1995, 2000; Das et al., 1997; Davis and Haissig, 1994; Hartmann et al., 1997).

Though exact mechanism of physiological response remains un-cleared (especially in mangroves), the biochemical studies on root initiation indicate that the root inducing effects of the auxin treatments, that is, IBA and NAA were related to the variation of total soluble sugar,

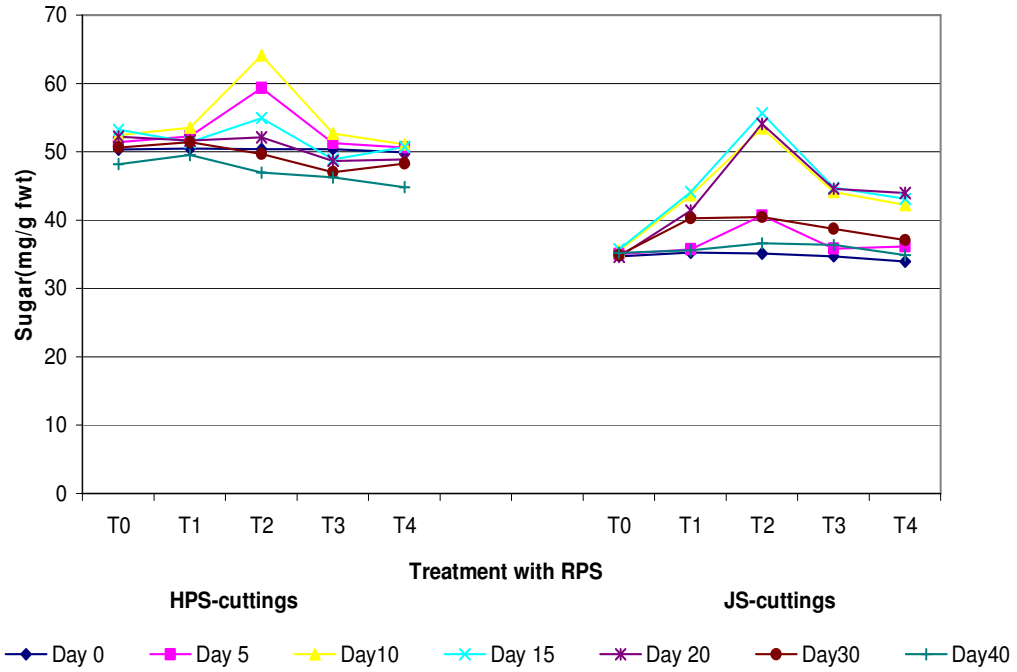

Figure 15. Sugar changes during rooting period in HpS (left) and JS-cuttings (right) in response to RPS.

starch and protein. It could be suggested that available carbohydrates are utilized during adventitious root induction and development in both types of cuttings. Irrespective of types of cuttings and treatments, total soluble sugar and starch contents in rooting zone increased up to 10 - 15 days of treatment and decreased there after, which indicate mobilization of stored sugar and starch by the activity of hydrolytic enzymes and translocated to the rooting zone of cuttings to act as source of energy for cellular division and differentiation (Hartmann et al., 1997; Nanda, 1970; Haissig and Davis 1994; Husen and Pal, 2007). The trend of fluctuation in the protein content during rooting was also found same as in sugar and starch. A similar trend in protein utilization during adventitious root regeneration in cuttings of several other species has also been reported. The 'max value' of sugar, starch and protein content had been noted at the time of induction of root primordia (10 - 15 days after treatment) which was evident in the present anatomical study. These findings further corroborated with Husen and Pal (2007).

Treatment of shoot cuttings with NAA or IBA increased levels of total soluble sugar and starch in the rooting zone. Numerous investigators have reported that increase in sugar content in the rooting zone of cuttings caused by auxins which may be attributed to an increase in starch hydrolysis (Nanda, 1970) and/or increased sugar transport towards rooting (Altman and Wareing, 1975; Middleton et al., 1980; Haissig, 1982). Moreover, auxin–carbohydrate interactions are also observed to be vital for rooting (Das et al., 1997; Nanda, 1970; Andersen et al., 1975; Veierskov and Andersen, 1982).

Conclusion

In conclusion, it could be suggested that the juvenile stem cuttings (with or without hypocotylar part) of *A. corniculatum* are capable of producing adventitious roots for vegetative propagation. Better rooting response (in terms of % rooting and root number per cutting) may be obtained in hypocotylar juvenile stem cuttings. Application of RPS (IBA1.0 mg/l plus NAA 5.0 mg/l) enhanced maximum rooting process which is evident anatomically as well as biochemically. Hence, this new approach of using mature hypocotyls (to be collected before havoc destruction by predators) as explants for mass vegetative propagation, may reduce the conventional use of stem branches of adult and reproductive tree members and thus restrict further depletion of natural population.

ACKNOWLEDGEMENTS

We would like to thank the Ministry of Environment and Forests (MoEF), Government of India for financial assistance vide project No.10/86/2004-CS/BG. We are also thankful to the Department of Forest and Environment (Mangrove Wildlife Division), Government of Orissa for providing necessary help during field work in the mangrove ecosystems of Bhitarkanika National Park,

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