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Axillary Mass Multiplication *in vitro* And Tolerance towards Salt Sensitivity in *Acmella oleracea*

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INTRODUCTION

Acmella oleracea Linn (Asteraceae) is a tropical herb with a growing height of 30-50 cm). It is found in moist areas with bright light available and blooms in mid- summer [2]. The golden buds with a rust-red center look like an eyeball [21] so the plant is also named as an 'Eye Ball Plant'. Plant has anesthetic and bacteriostatic properties and help to fight tooth lesions and decay. Its balls are used to treat ailments like throat pain and constipation [6]. Tissue culture is now being commonly used for not only mass propagation of plants with medicinal potentials but they also provide suitable plant models for studies related with stress plant physiology due to strictly controlled growth conditions [18, 19, 3, 8]. Plants have developed various methods to cope with environmental stress such as pesticides, salt, heavy metals etc and any modulation in growth parameters could lead to increased sensitivity. Authors have used certain enzymatic as well as non-enzymatic growth parameters to observe the plant potential to carve the negative effects of salt stress. Also an efficient mass propagation protocol was established for optimum production of plantlets *in vitro* for its commercial uses.

MATERIALS AND METHODS:

Young plants of *Acmella* were collected from nearby areas of Forest Research Institute, Dehradun and nodal explants with axillary buds were taken from young & healthy plants for culture initiation. Explants were surface sterilized using 3-4 drops of Tween-20 for 3-4 minutes followed by surface disinfection with 0.2% $HgCl_2$ (w/v) for 3 minutes [11, 10, 12, 27]. The nodal segments were then inoculated under aseptic conditions on Murashige and Skoog's (MS) medium [18, 19] with slight modifications (Manganese sulphate dihydrate was used in place of Manganese sulphate tetrahydrate) and concentration of $Na_2EDTA.2H_2O$ was reduced to 29.2 mg/l in place of 37.25 mg/l.). The medium was supplemented with usual salts and vitamins and 2.8% sucrose (w/v; Hi- Media), 100 mg/litre myo-inositol (E. Merck) and 0.8% agar (w/v; Hi-Media).

Media were supplemented with various concentrations of BAP (6-benzylamino purine) alone and in combinations with NAA (α- naphthalene acetic acid) and IAA (Indole-3-Acetic acid). The pH of the media was adjusted to 5.8. The cultures were kept at 25± 2^0C under illumination with white fluorescent tubes (50 µM $m^{-2}s^{-1}$) at 82% relative humidity. They were maintained under light for 14 hours followed by 10 hours dark period. Each treatment was used in replicates to avoid manual errors and to get repeated accuracy in results.

Sprouting of axillary buds was seen on nodal segments after 15-25 days of culture (Figure 1 a). These buds, with part of the growing nodal segments, were subcultured on modified medium supplemented with BAP (1.30-4.40 µM) + IAA (1.40-2.30 µM) or NAA (0.44-1.33µM) for further shoot multiplication. Nodal explants (0.7-0.9 cm) from the axenic shoots were recultured on agar solidified medium containing different concentrations of BAP, NAA and IAA. Gibberellic Acid was used for elongation of shoots (GA_3, 0.15-3.10 µM). Roots were then induced in shoots that attained the height of 2 cm± 0.5 cm by transferring to MS medium supplemented with different combinations of IAA, and NAA. The roots were initiated in rooting medium as well as in basal medium. The roots produced in basal medium were observed to be thin with lesser viability.

Eight weeks old plantlets were transferred to pots containing sterilized soil and sand mix (1:1), covered with polythene bags with perforations, for 10 days and the pots were kept below 25±2^{o}C, for acclimatization. These were then transferred to green house, after removing polythene covers, for hardening [25, 26]. Plantlets (28 days old) were subjected to the salt treatment. They were placed first in liquid nutrient media for 72 hours (Knops solution) and then subjected to various concentrations of NaCl. Each experiment was conducted in triplicates.

Extraction and estimation of total Protein, Malondialdehyde and peroxide contents

Growth determination was done by estimating total protein contents after 15 days of treatments. Protein contents were determined using Folins-Lowry method with lysozyme as the standard [16]. Content of hydrogen peroxide radicals was estimated by using method of Sagisaka (1976) [24]. The level of lipid peroxidation was measured in terms of total malondialdehyde (MDA) contents. The reaction reagent consisted of 0.4 N TCA + 19.68 ml of distilled water + 0.4 ml of HCl + 100mg TBA [14]. Prepared leaf extract (in phosphate buffer) was added to the reaction reagent and absorbance was taken at 532 nm.

Extraction and Estimation of Flavonoid and proline contents

Flavonoids were extracted in salt-treated and control plants by using the method of Mirecki and Teramura (1984) [17]. Extraction mixture consisted of acidified methanol (methanol: water: HCl, 78: 20: 2, v/v) + leaflets, incubated for 24h at 4^0C. The filtered extract was then used for measuring the absorbance at 320 nm [17]. Flavonoid contents were expressed as absorbance g^{-1} fresh

mass of tissue at 320 nm. Proline contents in leaf homogenate of salt- treated and untreated cultures were estimated according to the standard method [4]. Proline contents in unknown samples were calculated by comparing with standard curve of L-proline. Amount of proline is represented in terms of µg g^{-1} FW.

RESULTS AND DISCUSSION:

Best induction of multiple shoot formation from leaf explants occurred on medium containing BAP (3.40 µM) and IAA (2.50 µM). Amongst different combinations of plant growth substances used, maximum shoot regeneration per explants was found to take place with BAP (3.5 µM), NAA (2.20 µM) & IAA (1.5 µM) (Figure 1 b and b) with about 30 shoots in 50 days. A combination of BAP (1.85 µM) + IAA (1.3 µM) resulted in 22 shoots in 50 days. A high dose of BAP(>5 µM) and IAA (>4 µM) resulted in inhibition of shoot multiplication as well as induction of shoots.

Root initiation was tried with combinations of BAP, IAA and NAA, but best root growth was promoted by BAP (0.50 µM) used with IAA (1.75 µM, Figure 1 c) and with IAA alone (1.75 µM, Figure 1 d). It was observed in the present investigations that multiple plant regeneration from leaf explants of *Acmella oleracea* could be induced on MS medium. Plant multiplication rate was dependent on appropriate combinations of plant growth substances (PGSs). The current work provides preliminary information and methodology for rapid propagation of this valuable plant from leaf explants that might help in the improvement of conservation methods.

Overall growth in terms of protein contents and accumulation of proline and flavonoids was observed to vary with alterations in pH of the medium. Total protein contents decreased with low pH (% control decrease was 18% on 4.5 pH). Further with the increase in pH, protein contents were slightly increased (% control increase = 10-25%) but at highly alkaline pH (9.8), it was reduced to 10% with compared to the control (Fig 2 a). Emergence of anti-stress proteins with respect to the modulations in proton stress may be responsible for observed changes as depicted by earlier authors in other plants too. Initial recovery might be due to the increased pool of enzymatic as well as non-enzymatic antioxidants in cells [1].

Cells contain important non-enzymatic antioxidants such as carotenoids, flavonoids, proline, glutathione, α-tocopherol etc. that help in mitigating the toxic effects of free radicals and active oxygen species under oxidative stress. Flavonoid is assumed to play an important role in overcoming the oxidative stress in cells [22]. Evidences suggest that the presence of flavonoid in UV-B irradiated leaves could alter the perception or response of other defense mechanisms. Presently, flavonoid contents showed remarkable 35% decrease in the Flavonoid contents at highly acidic pH (4.5). But 8-17% enhancement in contents was observed with increase in pH (6 to 8). 20% reduction at pH 9.5 showed the sensitivity of antioxidant and its potential to reduce oxidative stress (Fig 2 b). Since Flavonoid inhibits the enzymes responsible for superoxide anion production thus the increase in their values may be attributed to the protection from free radical induced damage.

A high accumulation of proline contents (18-45%) was observed during the modulations in proton stress. The accumulation and protective effect of proline has been observed in many higher plants and bacteria as well as plants [5]. Stress induced lipid peroxidation of the cellular components in plants were studied by estimating the level of MDA in treated and control plantlets and the related data are depicted in the fig 2 b. The lipid peroxidation in non- stressed plantlets was observed as 1.16 nmol MDA (mg fresh mass)-1, whereas it was recorded to be 1.89, 1.22, 2.36 and 2.89 nmol MDA (mg fresh mass)$^{-1}$ with pH 4.5, 6.8, 9.5 nmol MDA (mg fresh mass)$^{-1}$respectively (Fig 2 d). A high level of lipid peroxidation in stressed plants is indication of high oxidative stress condition. Similarly the increase in peroxide radical contents was observed to be linearly related with the level of lipid peroxidation (Fig 2 c). MDA is an intermediate compound produced due to lipid peroxidation; the measurements of its contents can be used as an index for the injury caused by free radicals produced during oxidative stress. The results obtained here are in agreement with other authors [1, 13].

It is evident from the results obtained that high level of proton stress may affect the overall growth of plant species. Growth retardation in term of protein contents is indicator of plant specific requirement of salinity or alkalinity in soil. High level of lipid peroxidation and increase in peroxide radicals are suitable measures of damage caused due to free radical induced oxidative stress in plants. Elevation in the contents of non enzymatic antioxidants in cells is also an indicator of boost up in antioxidant machinery since these provide protection to plants from various stresses. *Acmella* is high value medicinal plants and its mass propagation strategy can be used further in suspension cultures to obtain suitable secondary metabolites for commercial uses. It is widely used in many traditional medicines prescribed under different systems of medicine. *Acmella* species have long been used as traditional medicine for local anesthetic, antibacterial [23], antiviral, antihypertensive, larvicidal and diuretic actions [20]. The whole plant, leaves, and roots are used for a variety of purposes in many herbal medicines. For example, the leaves are used to cure throat infections and for the treatment of ulcers [6, 7]. It is shown that the plant is being used traditionally in treatment of several respiratory diseases. It is, therefore, important to maintain a balance between its use and conservation status. Many researchers have paid attention in this direction. Suspension cultures widely used for the *in vitro* production of secondary metabolites using large and small scale fermenters, proved the importance of tissue culture technology [9, 15]. Current study supported the optimized rapid multiplication in *in vitro* conditions and use of raised plantlets as model organism for stress physiology experiments.

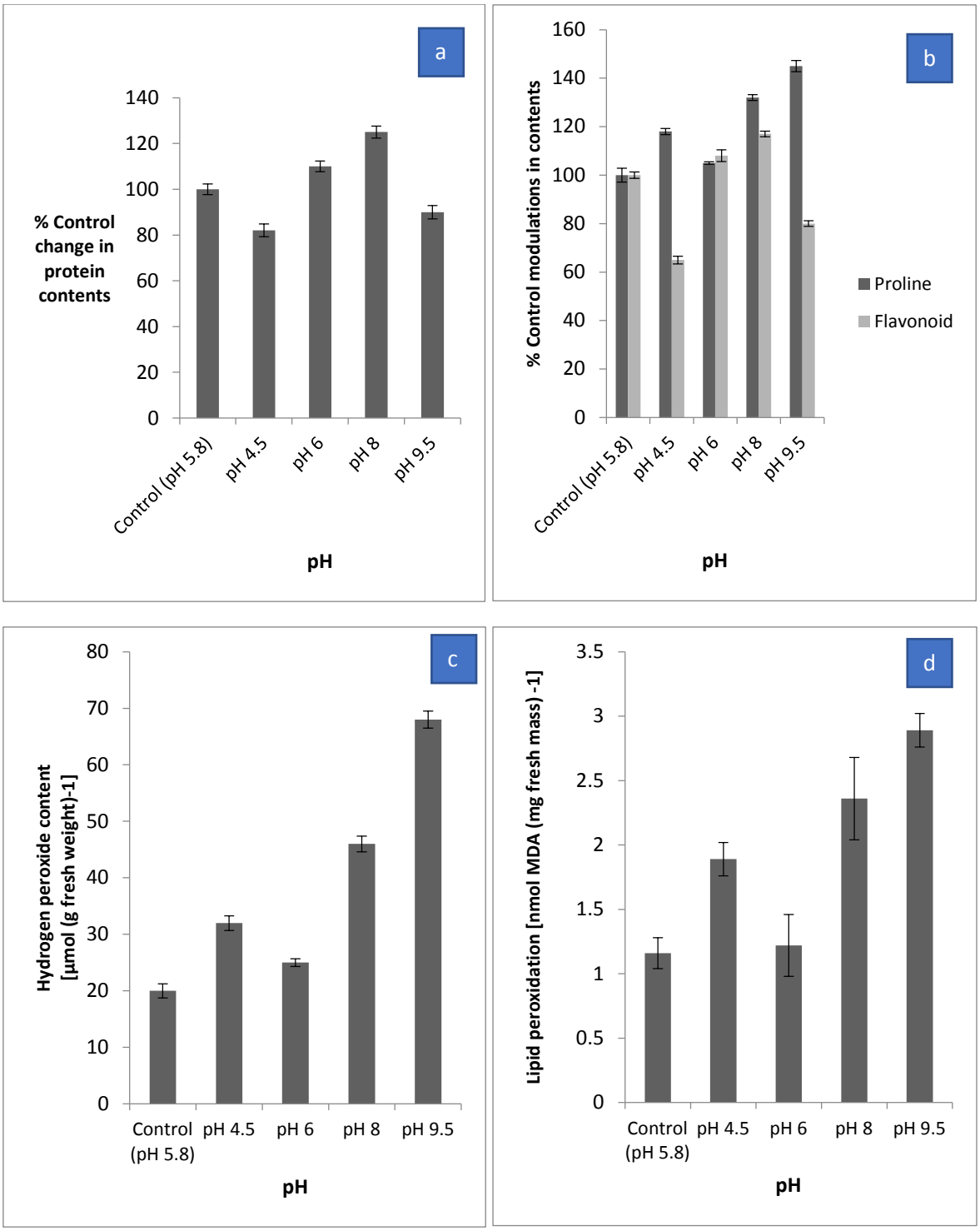

Figure 2 Modulation in Biochemical profile of *in vitro* raised plantlets with respect to proton stress. Alterations in contents of protein (a), proline, Flavonoid (b), hydrogen peroxide radicals (c) and Lipid peroxidation in term of total MDA produced (d) are clearly evident from the studies.

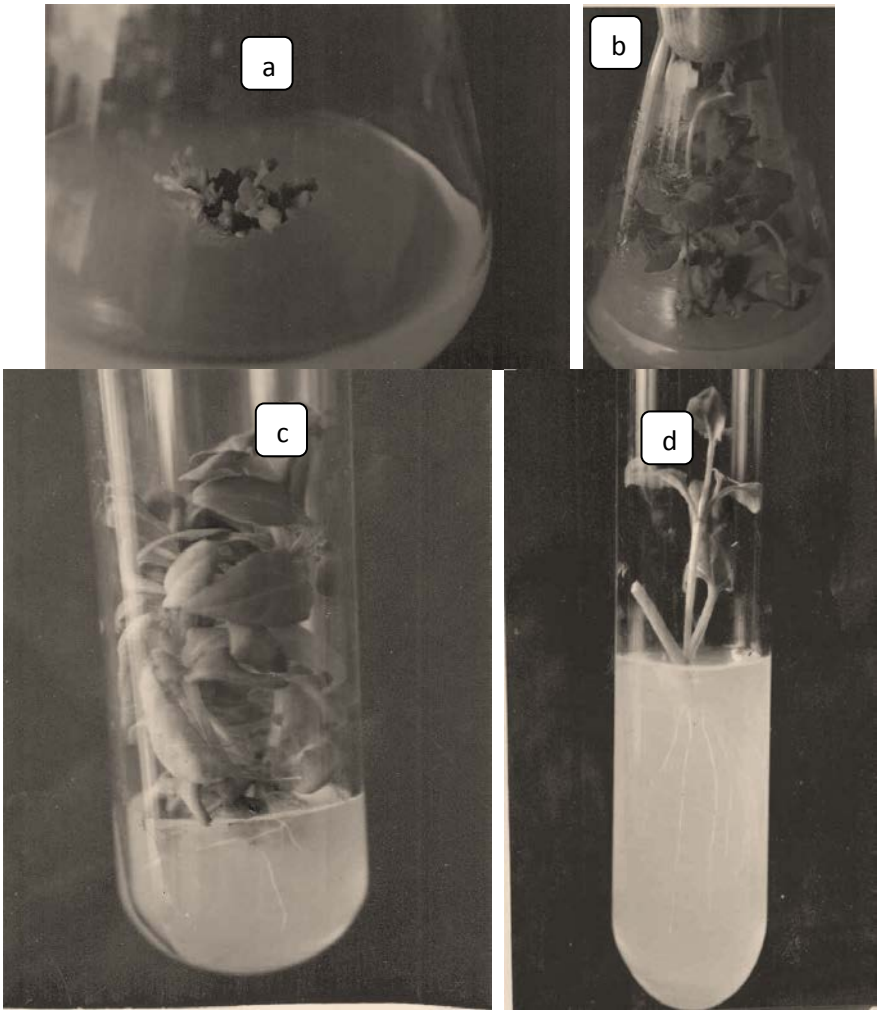

Figure 1 *In vitro* mass propagation of *Acmella Oleracea* (Nodal proliferation (a), shoot multiplication (b) and Root formation (c and d)

Conflict of Interest:

The research work has been carried out as an partial fulfillment of Doctoral research work of first author (Mr Shivraj Gautam) under the guidance of Dr Rina Rani Singh (Supervisor) and Dr Santosh Kumar Singh (Co-Supervisor). The authors wish to declare that "There is no conflict of interest among any of the author and associated Institutions".

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