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Research Article

Induction of Somatic Embryos from Mature Embryo Culture under Abiotic Stress and Estimation of Proline Status in a Millet Crop, *Paspalum scrobiculatum* L.

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ABSTRACT

This study reports the establishment of embryogenic callus from mature embryo culture of millet (*Paspalum scrobiculatum* cv. PSA_1) under abiotic stress (water, heavy metals and salinity) conditions and simultaneously the proline contents of the embryogenic callus induced under stress conditions were also estimated. To begin with, mature embryos were cultured on MS (Murashige and Skoog, 1962) medium supplemented with 2,4-dichlorophenoxyacetic acid or 2,4-D and optimal frequency 37% of embryogenic callus was obtained on MS medium supplemented with 2,4-D (20µM) which eventually germinated into plantlets on 2,4-D-free basal medium. Further, for study on water stress treatments, mature embryos were cultured on MS medium supplemented with 2,4-D (20µM) and various concentrations of PEG (2.5, 5.0 and 10%) or mannitol (0.5,1.0 and 2.5M) separately. Results indicate that the treatment of PEG at concentration of (5%) supports somatic embryogenesis whereas mannitol at concentration of (2.5M) proved to be inhibitory. In case of heavy metals (Co, Zn and Ni) stress treatments at various concentrations (100, 250 and 500µM) of each along with 2,4-D (20µM), Co and Zn generally proved to be inhibitory for somatic embryogenesis, however, Ni at high concentration (500µM) was effective to induce embryogenic callus even on 2,4-D-free medium. Moreover, for salinity stress treatments on various concentrations of NaCl (50, 100 and 250mM) along with 2,4-D (20µM), the responses were influenced by the concentration of salt present in the culture medium. High concentration (250mM) of NaCl proved to be lethal for embryogenic responses. During this study, accumulation of endogenous proline content in calli grown under abiotic stress was also analyzed and it was found that proline concentration in embryogenic callus was generally based on concentration of stress causing agents in the medium and exposure durations of the tissues.

Key Words: Mature embryo, Callus**,** Somatic embryo, Abiotic Stress, Proline, Millet

[1] INTRODUCTION

Millets are staple foods that supply a major portion of calories and protein to large segments of populations in the semi-arid tropical regions of Africa and Asia [1]. *Paspalum* is a genus of the grass family gramineae and most of them are tall, perennial

grasses. They are warm-season C_4 grasses and are most diverse in subtropical and tropical regions. *Paspalum scrobiculatum* L. is traditionally used to treat diabetes mellitus and it is recommended as food for diabetic patients [2]. Aqueous and ethanol extracts of this grain produced a dose-dependent fall in fasting blood glucose (FBG) and a significant increase in serum insulin level. This indicates that *P. scrobiculatum* possesses significant anti-diabetic activity [3, 4].

Somatic embryogenesis is an important approach for *in vitro* regeneration technology and it can be highly significant for its application in plant improvement programs. It is the most preferred method of plant regeneration and moreover, due to the presence of well-developed root and shoot primordia, somatic embryos germinate easily to produce plantlets without an additional step of rooting [5]**.** This study reports the use of mature embryo tissues as explants for somatic embryogenesis and regeneration and it provides remarkable advantages as compared to immature tissues such as leaf-base or immature embryos because these are available in unlimited quantity and also don't cause seasonal influence on *in vitro* response.

Over the years several approaches, including utilization of somaclonal variations [6] and conventional plant breeding strategies were used to develop high-yielding millet varieties. Simultaneously, environmental stresses such as drought stress and presence of heavy metals and salt in soil are the major abiotic factors that limit crop productivity in terms of quality and quantity both. Since, the major areas of cultivation of these varieties are rain-fed, therefore, the potential yields are affected by severe drought stress [7, 8] and drought induced oxidative stress [9]. Moreover, salinity stress also reduces potential yields of the crops [10, 11]. More specifically, seed germination and seedling establishment stages are highly susceptible to drought and salinity stress [7, 11].

Although, in the direction of millet crop improvement, various agronomic practices during sowing and plant establishment in the field [12, 13] were brought in practice but have not improved the productivity of this millet [7]. Therefore, developing plants having higher intrinsic stress tolerance is necessary for yield improvement [14]. In view to establish an alternative approach to achieve stress tolerant plants, this study aims the *in vitro* induction of somatic embryos from mature embryo culture under abiotic stress conditions which is required in the selection of crop cultivars for tolerance to abiotic stresses.

Additionally, evidences indicate that proline may act as an osmo-protectant by interacting with crucial macromolecules of the cell and modulating their biological activity [15] and the high levels of proline accumulation in stress exposed tissues are believed to protect plant tissues against stress by acting as N-storage compound, osmo-solute and hydrophobic protectant for enzymes and cellular structures [16]. Thus, a parallel investigation during this study was also undertaken to establish a correlation for induction of somatic embryos on exposure of abiotic stress causing agents along with accumulation of endogenous proline level during different doses and durations of stress treatments under *in vitro* conditions.

[II] MATERIALS & METHODS

2.1 Procurement of explants and nutrient medium formulation

Mature seeds of *Paspalum scrobiculatum* L.cv. PSA_1 were procured from University of Agricultural Sciences, Bangalore (India) and were employed as source materials for isolation of mature embryo explants. Mature seeds were firstly dehusked and washed with

2% (v/v) detergent solution 'teepol' for 15 minutes and later were surface sterilized with 70% ethanol for one minute. It was further followed by washing of seeds with Sterile-Distilled-Water (SDW) and subsequent treatment with $HgCl_2$ (0.1%) for 10 minutes. Treated seeds were then rinsed repeatedly 3-4 times with SDW to remove out traces of adhered $HgCl_2$ from seed surface.

Thereafter, sterilized seeds were soaked in SDW for 3 days in dark at room temperature to allow partial emergence of mature embryos from the swollen seeds. At this stage, mature embryos were dissected out from the endosperm without damaging the radicle portion. Aseptically excised mature embryo was cultured in the nutrient medium where embryogenic side was kept down in the contact of medium.

The basal nutrient medium employed in present study was consisting of MS [17] salts and vitamins. Mature embryos were inoculated on hormone-free MS basal medium or medium supplemented with hormone 2,4-D at various concentrations (10, 20 and 50µM) along with sucrose 2.5% (w/v). All nutrient components were mixed together followed by adjustment of pH 5.8 and finally solidified with agar (0.8% w/v) prior to autoclaving at 121^{o}C for 20 minutes.

2.2 Callus induction and differentiation of somatic embryo

Cultures were incubated at 25^{o}C in dark for a week followed by light incubation and observed weekly under a stereomicroscope. Primary callus with or without embryoids after two weeks was sub-cultured on the same medium for proliferation and differentiation of somatic embryos followed by further subsequent maintenance by weekly sub-cultures. The frequency of embryogenic callus formation was recorded stereo microscopically after two and four weeks of culture initiation.

2.3 Plantlets regeneration

After 4-5 weeks with two subcultures, embryogenic calli were transferred to 2,4-D-free basal medium for regeneration and these cultures were incubated for another 1-2 weeks under light conditions. At the end of another two weeks, most of these calli exhibited regeneration of plantlets.

2.4 Abiotic stress treatments

Mature embryos excised from the soaked seeds were also cultured under various abiotic stress (water, heavy metals and salinity) for the induction and differentiation of somatic embryos.

2.4.1 Water stress

Treatments of polyethylene glycol (PEG 6000) and mannitol were given in the 2,4-D containing callus induction medium for three weeks and then transferred to basal medium without 2,4-D but the stress treatments were continued in the basal medium for another two weeks. Explants not to exposed to the stress treatment on the basal and on the 2,4-D media served as controls. PEG was supplemented in the primary callus induction medium at various concentrations (2.5%, 5% and 10%) along with 2,4-D (20µM). Similarly, in another set of experiments, mannitol was included in the induction medium at a concentration of (0.5, 1.0 and 2.5M) supplemented with 2,4-D (20µM).

2.4.2 Heavy metals stress

Mature embryos were cultured on callus induction MS medium supplemented with heavy metals (Co, Zn and Ni) at a concentration of 100µM, 250µM and 500µM each either alone or in presence of 2,4-D (20µM) and further the same procedures like water stress were also followed for heavy metals stress treatments.

2.4.3 Salt stress

To obtain tolerant callus against salt stress, mature embryos were inoculated on MS

medium supplemented with various concentrations of NaCl (50mM, 100mM and 250 mM) along with 2,4-D (20µM). In each concentration of NaCl, calluses were grown for at least three consecutive passages and same steps for sub-cultures and observations were followed here again.

2.5 Statistical analysis

For each treatment during establishment of embryogenic callus and stress experiments, 25 tubes were raised and each experiment was conducted two times. The medium was changed at every 10-12 days of intervals. The data on callus induction efficiency measured as the number of calli or embryogenic calli obtained/ total number of mature embryos cultured x 100.

For determination of proline content in mature embryo derived calli induced under various stress treatments, techniques of Bates et al. [18] were employed in present study.

[III] RESULTS

3.1 Establishment of embryogenic callus

On control MS basal medium, mature embryos germinated quickly and within a week formed young seedlings whereas explants cultured on 2,4-D supplemented medium started to exhibit tendency of slow germination followed by initiation of callus formation. Further, these young shoots and roots from germinating embryos were cut out completely and the callus responding explants were cultured again on the same culture medium for callus proliferation.

The basal medium supplemented with 2,4-D (20µM) was found to be optimal (37%) for the induction of embryogenic callus (**Fig. 1A**) from already formed primary callus when incubated for one week in the dark followed by 2-3 weeks under low light. An increase of 2,4-D up to 50µM as in medium favored the formation of non-embryogenic callus (**Fig. 2**). The successive sub-cultures gave rise to the formation of numerous clusters of typical embryoids **(Fig. 1B & C)** at different developmental stages. Moreover, plantlets were also achieved by precocious germination of already matured somatic embryos even on 2,4-D supplemented medium or on 2,4-D-free basal medium (**Fig. 1D**) at the end of 6-7 weeks of culture initiation.

3.2 Effect of abiotic stress on somatic embryogenesis and proline content

In present study, mature embryo explants have been subjected to various abiotic stress treatments (water stress, heavy metals and salinity stress) to basal MS medium with 2,4-D (20µM) to analyze the efficacy of stress on induction and differentiation of somatic embryos after second and fourth weeks of culture initiation. Simultaneously, efforts have also been made in present study to analyze the changes, if any, in proline content status in the mature embryo derived embryogenic callus induced under various concentrations and durations of stress treatments at the end of second and fourth week of culture initiation.

3.2.1 Effect of water stress

During this study, mature embryos were subjected to polyethylene glycol treatment at various concentrations along with 2,4-D in the induction medium. Significantly, the increase in percentage of embryogenic cultures at 5.0 % concentration of PEG (**Fig. 3**) was recorded and this frequency was higher than the control (37%). This response was more evident when PEG was present in the basal medium during the embryo differentiation phase at the end of four weeks of culture initiation. However, with the increase in concentration of PEG, frequency of embryo forming cultures gradually declined and at high concentration of PEG (10%), embryogenesis frequency was recorded to be minimal (11%).

Additionally, another osmo-regulatory agent mannitol was selected for water stress during this study and when mannitol was present in the induction medium at concentrations of 0.5, 1.0 and 2.5M, percentage of embryoid formation declined after four weeks. In this case, maximum frequency of embryogenesis (17%) was recorded as compared to mannitol-free control and PEG- treated calli (**Fig. 3**). Furthermore, when the mannitol treatment was continued at 2.5M for four weeks, very low 2% callusing could be seen and such calli eventually failed to differentiate somatic embryos and gradually necrosed.

The endogenous proline status was analyzed during stress treatments and it was found to increase with increasing concentration of PEG in the medium at the end of two and four weeks of culture initiation (**Fig.4).** In case of embryogenic calli developed at higher concentration of PEG (10%) for four weeks, endogenous proline levels were found to increase (up to 7-8 folds as compared to the control).

In comparison to PEG treatment, the endogenous proline accumulation in case of mannitol stress was found to be much higher. Up to 18 folds increase in proline levels were observed when the explants were treated with mannitol at higher concentration (2.5M) as compared with the control on 2,4-D (20µM) containing medium (Fig. 4). When this treatment was continued in the medium for four weeks, the increase was much evident (183µmoles/g) than the control (10µmoles/g).

3.2.2 Effect of heavy metals stress

When the heavy metal ions copper, zinc and nickel were provided at 100µM, 250µM and 500µM levels each either alone or in combination with 2,4-D (20µM), there was general tendency of inhibition of callus growth and also in frequency of somatic embryogenesis with the increasing concentrations of heavy metals in the medium. All of them displayed a general inhibition of somatic embryogenesis (**Fig. 5**) and moreover, cobalt proved to be the least effective for morphogenic responses followed by zinc and nickel.

When these metals were applied without 2,4-D addition, surprisingly, only nickel could support induction of somatic embryogenesis but at lesser frequency whereas other metals cobalt and zinc failed to support somatic embryogenesis. The fact that nickel alone could cause induction of somatic embryogenesis was quite intriguing. Nickel was subsequently tried over a concentration range and 500µM (in the absence of 2,4-D) was found to be effective for induction of somatic embryogenesis almost equal to control (**Fig. 6.**). Moreover, a study on time requirement for embryogenesis revealed that nickel treatment for around two weeks exhibited the maximum response and further incubation was proved to be inhibitory.

Proline content level **(Fig.7)** was found to be maximum (272µmoles/g) in calli growing on nickel at high concentration (500µM) along with 2,4-D (20µM) supplemented medium at the end of fourth week of culture initiation. However, in 4-week-old calli growing on zinc supplemented medium (100µM), the proline concentration was recorded to be the minimum (56µmoles/g).

3.2.3 Effect of salt stress

To obtain embryogenic callus under salinity stress, mature embryos were cultured on MS medium supplemented with various concentrations of NaCl (50 mM, 100 mM and 250mM) in presence of 2,4-D (20µM). Growth of tissue was identified by the naked eye observation. Study on growth, tissue viability and proline content of control and treated calluses of NaCl revealed that growth value

of salt treated callus was less than that of the control one (Result is not given).

Almost 37% and 30% of embryogenic callus formation was observed in MS medium in case of control and treated cultures respectively. In general, the presence of NaCl in high concentration (250mM) was always inhibitory for embryogenesis in comparison to lower concentrations of NaCl (50 mM). Significantly, the maximum frequency of embryogenic callus formation (30%) was observed at lower concentration of NaCl when medium was supplemented with 50mM NaCl (**Fig.8**). There was general observation that with the increase of NaCl concentration in the nutrient medium resulted in inhibition in explants potentials for callus formation followed by reduction in somatic embryogenesis frequency and therefore, at higher concentration of NaCl (250mM), the frequency of somatic embryogenesis was found to be very low (4%).

During salinity stress treatments, an increase in free proline content has been observed in all *in vitro* grown embryogenic calluses with increasing NaCl in the medium. Free proline content of the calluses revealed that NaCl (250 mM) treated calli contained at least several times more endogenous free proline than control (**Fig. 9).**

[IV] DISCUSSION

Establishment of a stable *in vitro* regeneration protocol is an important component of any genetic transformation protocol because it provides sources of competent explants that can be used as recipient of introduced foreign genes. Any regenerable cell, tissue, or organ can be used as explants during *in vitro* regeneration studies. In literature, the efficient and stable plant regeneration protocols for all the important cereals and millets have been documented that are mainly based on application of immature tissues as a source of explants like young inflorescence [19], immature embryo [20] and leaves, root [21]. Here, in present study on a millet crop, *Paspalum scrobiculatum*, mature embryo was employed for establishment of embryogenic callus which could be further a suitable target tissue for introduction of desired genes to get genetically improved verities of this millet.

In previous study on *Paspalum scrobiculatum*, the requirement of 2,4-D for induction of somatic embryos has already been optimized for 11-days on induction medium [22] and thereafter somatic embryo development takes place on a basal medium (differentiation medium). During this study, 2,4-D at (20µM) proved to be most competent inducer for induction of optimal frequency of somatic embryogenesis from mature embryo explant.

Efficacy of abiotic stress on somatic embryogenesis and proline status

This study also reports to evaluate the correlation in between modes of abiotic stress treatments and cellular potentials of mature embryo for somatic embryogenesis. Stress-induced somatic embryogenesis has been reported in many plants. In carrot, 2,4-D functions as an auxin and a stress inducer [23]. Of the various conditions conducive for somatic embryogenesis, osmotic stress, dehydration stress, heat shock stress, salinity stress and heavy metal ion stress are the most potent stressor [24, 25].

Polyethylene glycol (PEG), a non-penetrating osmotic agent that lowers the water potential of the medium, has been used extensively to stimulate drought stress in plants [26]. Results obtained during this study on millet is in conformity of earlier report on wheat leaf base culture [27] where PEG also proved to be equally good at low concentration (2.5%) in order to stimulate somatic embryogenesis. From this results, it is inferred that PEG acts

synergistically with 2,4-D for somatic embryo induction and differentiation [27]. Such a PEG stimulated enhanced regeneration *in vitro* has been reported in rice [28] and other crops but has not been reported in millets so far.

Mannitol neither supports *in vitro* tissue growth nor is it metabolized by higher plants. In comparison to PEG, mannitol did not stimulate somatic embryogenesis at any concentrations in present study. Moreover, high level of mannitol in the medium even proved to be inhibitory for callus induction. Such inhibitory effects of mannitol to stimulate somatic embryogenesis have been also observed in wheat leaf-base culture [27].

Water stress can result in induction of somatic embryogenesis and even enhance regeneration efficiency without the obligate need for exogenous hormones. Water stress in the form of drought or salt stress has been found to be promotory for regeneration by other workers as well in different systems [29-31]. Osmotin protein is shown to be associated with tolerance to water stress in plants by numerous studies. Overproduction of osmotin induces free proline accumulation in stressed cells. Moreover, synthesis of osmotin is induced by ABA which is involved in adaptation of cells to stress and is instrumental in osmotic adjustment [32]. In fact, it has been suggested that at low concentrations, osmotic stress may cause a disruption of plasmodesmatal interconnections between pre-embryonic cells, allowing cells to be physiologically isolated, thus aiding in a greater number of cells to differentiate [27].

Since, induction of somatic embryogenesis by 2,4-D was extrapolated to be similar to a stress response and various metal ions are reported to be effective. The heavy metals like Cu [33], Co [34, 35], Ni [35] and Cd [27] proved to be effective in stimulating morphogenesis in callus cultures. Although some heavy metals are widely used as micronutrients and very little is known about their effects on morphogenesis during tissue cultures [36].

In this study, the promontory effects of metal ions on the induction of somatic embryogenesis on mature embryo culture have been observed only with nickel. Cobalt and zinc both metals did not stimulate somatic embryogenesis at any concentrations even with 2,4-D, rather they proved to be inhibitory depending upon their concentrations and duration of exposures. Significantly, nickel could show stimulation of somatic embryos at high concentration with or without 2,4-D. However, at none of the concentrations of nickel, the frequency of somatic embryogenesis induced by nickel, was higher than that induced by 2,4-D without nickel.

In contrast, the promontory effect of nickel on somatic embryogenesis without 2,4-D on millet during this study is not in favor to the observation in case of *Setaria italica*, where Ni has been proved to be significant in order to stimulate somatic embryogenesis along with 2,4-D [36]. Also in carrot, stimulatory effects of heavy metals (cobalt and nickel) on somatic embryogenesis could be possible in the presence of auxin.

It is suggested that heavy metals help in the induction of high frequency morphogenesis closely linked to cell growth [35]. The increase in embryogenic frequency and growth could be possible in using Ni in the medium and at high concentration this growth was found to be arrested. This reduction in callus growth at high Ni could be due to binding of proteins [37] and regulation of ethylene biosynthesis pathways [38]. Moreover, the direct toxic action of Ni involves the reduction of carbohydrate supply and inhibition of mitotic activities [39].

Other heavy metals such as Ag and Cu also stimulated morphogenesis in wheat, triticale

and tobacco tissue cultures [33] and similarly in wheat leaf base culture where cadmium alone without 2,4-D was competent enough to induce somatic embryos [27]. However, regeneration from these embryoids on MS basal medium was not observed which is contrary to the observations made by Harada et al. [40] where in a better regeneration frequency was obtained by stress than by 2,4-D.

In general, decline in callus growth with the increase of NaCl concentration in the nutrient medium with or without 2,4-D observed in present study, is a usual phenomenon in millet and also in other plant tissues subjected to stress. This retardation of growth may be due to the fact that certain amount of the total energy available for tissue metabolism is channeled to resist the stress [41].

Proline has been known to play a critical role in osmoregulation in stressful environment in many plants systems and moreover, higher plants accumulate free proline in response to external salt and drought stress [42]. The results of proline accumulation during abiotic stress treatments during present study are in agreement with the earlier reports on the free proline accumulation under stress in seedlings, plants as well as in callus cultures [42]. Moreover, during stress in plants, there is the accumulation of certain organic metabolites includes sugar alcohols such as mannitol, sorbitol, trehalose etc. and free amino acids particularly proline compounds are reported to play a pivotal role in cellular osmotic adjustment in response to osmotic and salt stresses [43,42]. The importance of a mild osmotic stress in cell growth and plant regeneration *in vitro* is well documented [44].

The high levels of endogenous proline accumulation could be due to enhanced activity of ornithine amino transferase (OAT) and Pyrroline 5-carboxylate reductase (P5CR), the enzyme involved in proline biosynthesis as well as due to the inhibition of proline oxidase, proline catabolising enzymes [45]. To counteract the effect of increased accumulation of salt ions in the vacuoles, proline has been reported to increase in the cytoplasm, which might act as osmoticum [46].

Present results on sensitive cultivar PSA_1 of *Paspalum scrobiculatum*, indicate a concomitant increase in proline content with increase in concentration of stress causing agents in the nutrient medium are the first investigation of its type in millet and moreover, the stress induced increase in proline content more than that of the control is a clear indicator that this important millet crop can be regenerated and grown in adverse soil conditions.

[V] CONCLUSION

The results in present study on embryogenic regeneration from mature embryo culture of a sensitive millet cultivar of *Paspalum scrobiculatum* could be applicable to establish regeneration protocol for transgenic production for other millet crops in general and *Paspalum scrobiculatum* in particular. Furthermore, establishment of stable protocol for regeneration via somatic embryogenesis under abiotic stress treatments is a good technology of achieving abiotic stress tolerant cell lines which are required for crop improvement program.

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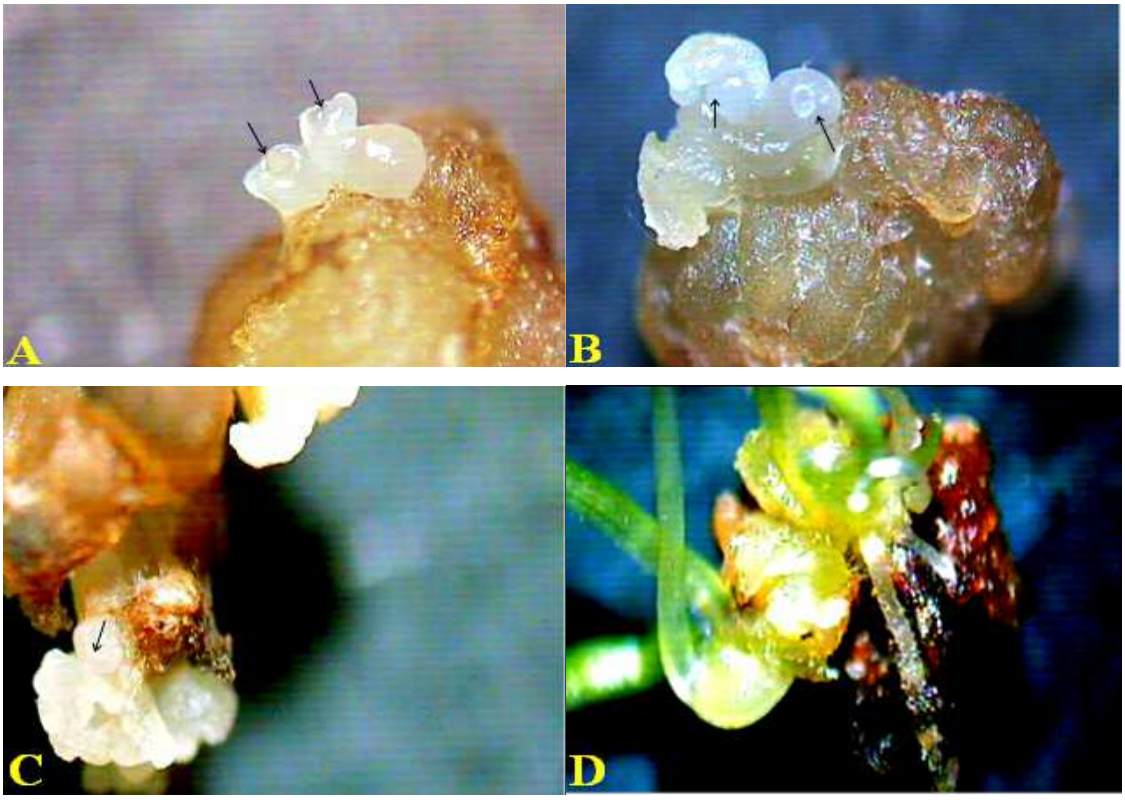
Figures:

Fig. 1 –*P. scrobiculatum*, Mature Embryo Culture.



A. 4-week-old-culture, showing differentiation of typical cup-shaped somatic embryos (Arrow Marked) on MS medium supplemented with 2,4-D (20µM).

B. 5-week-old-culture, showing differentiation of somatic embryos (Arrow Marked) on MS medium supplemented with 2,4-D (20µM) and PEG (5%).

C. 4-week-old-culture, showing differentiation of somatic embryos (Arrow Marked) on MS medium supplemented with nickel (500 µM) without 2,4-D.

D. 6-week-old-culture, showing germination of somatic embryos into plantlets on 2,4-D free- MS basal medium.

Fig. 2.*P.scrobiculatum*, mature embryo culture, showing frequency of callusing and somatic embryogenesis on medium supplemented with various concentration of 2,4-D.

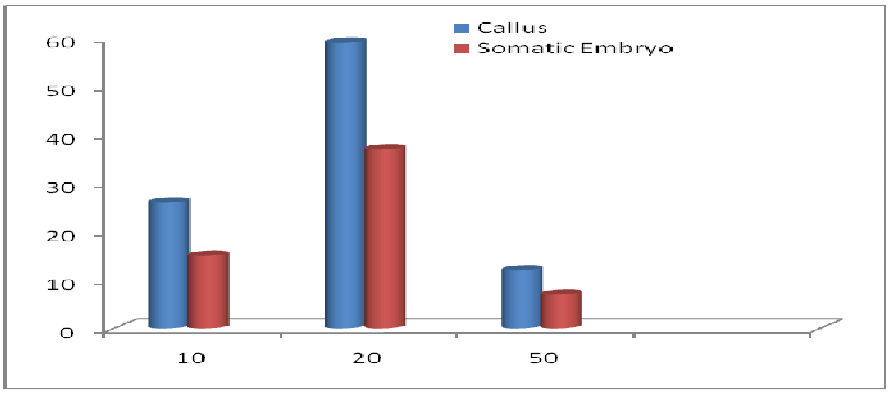


Fig. 3. *P.scrobiculatum*, mature embryo culture, showing frequency of embryogenic callus formation on medium supplemented with 2,4-D (20 µM) as control (Co) and also on medium supplemented with various concentrations of PEG and mannitol along with 2,4-D (20µM).

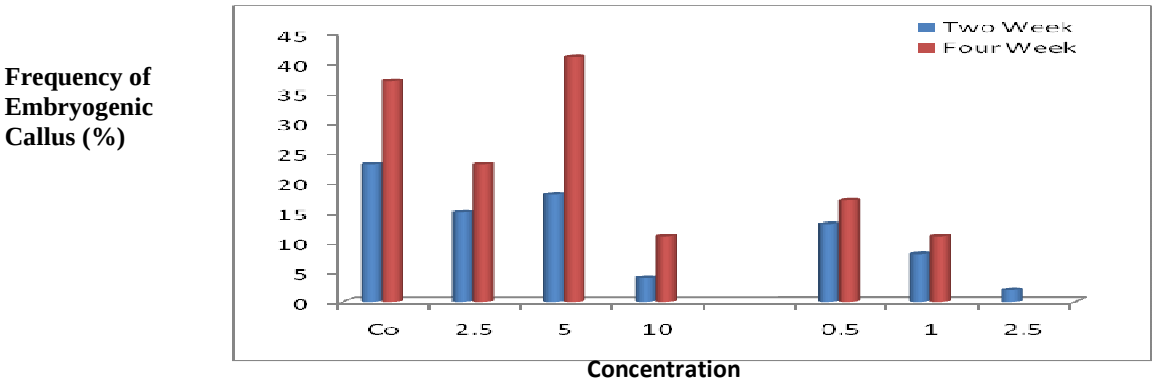


Fig.4.*P.scrobiculatum*, mature embryo culture, showing endogenous status of proline in embryogeniccalli grown on control (Co) medium and also onmedium supplemented withvarious concentrations of PEG and mannitol along with 2,4-D (20µM).

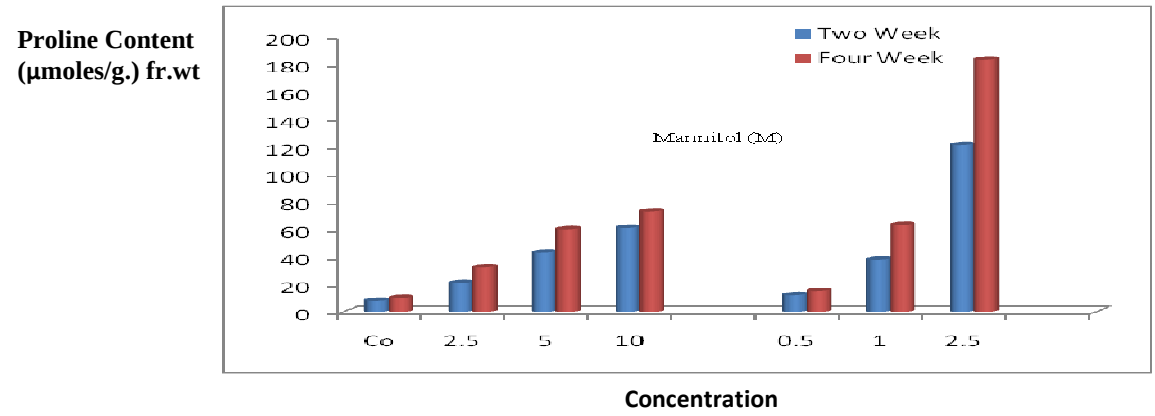


FIG.5.*P.scrobiculatum*, mature embryo culture, showing frequency of embryogenic callus formation on medium supplemented with 2,4-D (20 µM) as control (Co) and also on medium supplemented withvarious concentrations of cobalt, zinc and nickel along with 2,4-D (20µM).

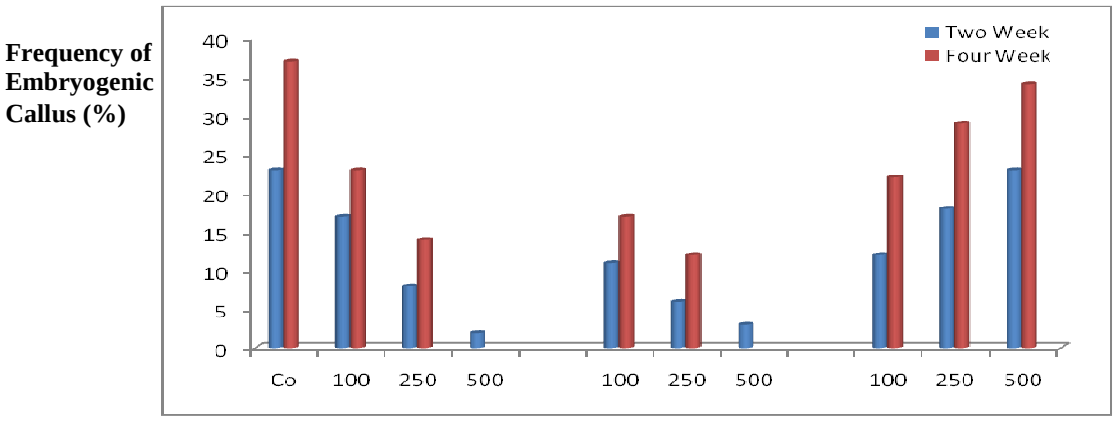


Fig. 6.*P.scrobiculatum*, mature embryo culture, showing frequency of embryogenic callus formation on medium supplemented with 2,4-D (20 µM) as control (Co) and also on medium supplemented with various concentrations of nickel alone without 2,4-D.

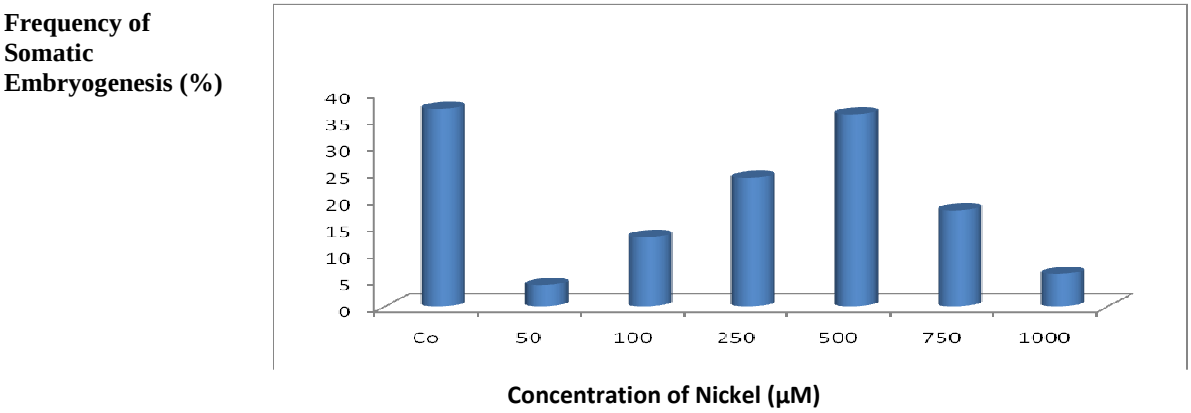


Fig. 7.*P.scrobiculatum*, mature embryo culture, showing endogenous status of proline in embryogeniccalli grown on control (Co) medium and also on medium supplemented withvarious concentrations of cobalt, zinc and nickel along with 2,4-D (20µM).

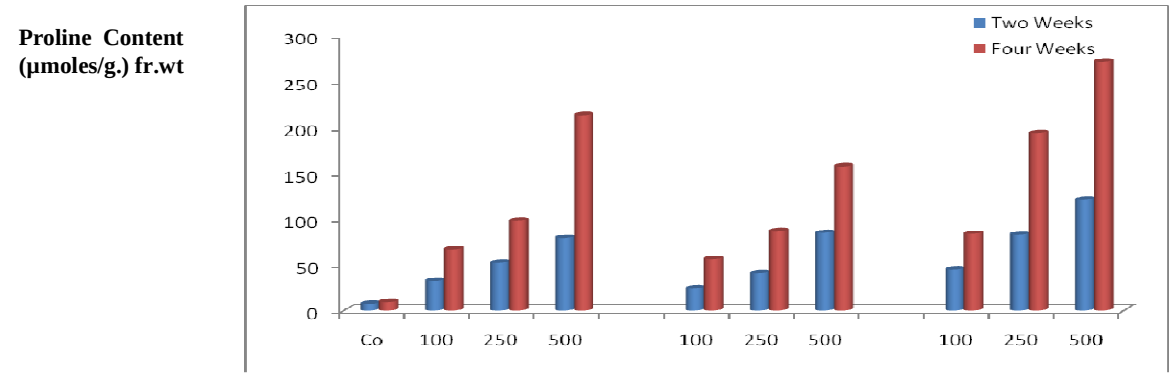


Fig.8.*P.scrobiculatum*, mature embryo culture, showing frequency of embryogenic callus formation on medium supplemented with 2,4-D (20 µM) as control (Co) and also on medium supplemented withvarious concentrations of NaCl along with 2,4-D (20µM).

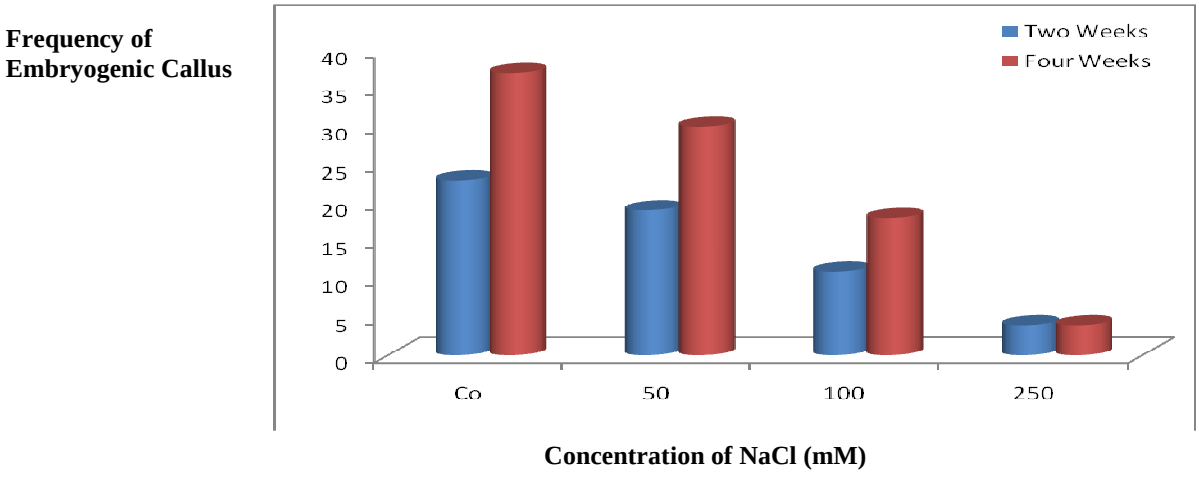


Fig. 9.*P.scrobiculatum*, mature embryo culture, showing endogenous status of proline in embryogeniccalli grown on control (Co) medium and also on medium supplemented withvarious concentrations of NaCl along with 2,4-D (20µM).