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INFLUENCE OF THIDIAZURON ON SOMATIC EMBRYOGENESIS AND PHYSIO-BIOCHEMICAL STUDIES OF *WITHANIA SOMNIFERA* L. DUNAL

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Using nodal segments, an effective approach for *in vitro* regeneration of a multi-medicinal plant-*Withania somnifera* via direct somatic embryogenesis was investigated. The embryogenic potency of nodal segments was tested on Murashige and Skoog (MS) medium augmented with various doses of thidiazuron (TDZ) (0.8-3.5 µM). 2.0 µM TDZ was found more potent than other concentrations as maximum rate of somatic embryos was recorded in this treatment. All the steps i.e. initiation, elongation and sprouting of somatic embryos from explants was achieved in the same treatment. About 85% of the somatic embryos germinated into complete plantlets. The plantlets were acclimatized successfully with 92% survival in greenhouse. Histological analysis confirmed that somatic embryos were protruded directly from the surface of explants. Different physiological and antioxidative enzymes activities were also analyzed at various period of acclimatization and showed positive signs towards the protection of micropropagated plants against stress developed during acclimatization to natural conditions.

Keywords: Biochemical, Histology, Micropropagation, Nodal segment, Thidiazuon

Withania somnifera L. Dunal commonly known as Ginseng and Ashwagandha,a reputed plant of Indian and African traditional medicinal system is a member of Solanaceae family. Withanolides are the major active constituents of *W. somnifera* that are isolated from its root and leaves such as withaferin, withanosides, withanine, somniferine, withananine and sitoindosides etc. Due to their presence, this plant is medicinally important and has been used in Ayurveda to relief anxiety and reduces stress and also to treat a number of diseases like arthritis, rheumatism, skin diseases, bronchitis, fever and inflammation etc. In recent years, many pharmacological studies suggested the effectiveness of *Withania* plant as anticancer and in cardiovascular activities (Kulkarni and Dir 2008). Besides, the therapeutic role of this plant has also been reported in central nervous system related disorders such as Alzheimer's disease, epilepsy, Parkinson's disease etc. (Tohda *et al. 2005).*

This plant is now being deleted from their natural habitat because of large scale unrestricted exploitation from the wild sources played a major role in its depletion. The International Union for Conservation of Nature and Natural Resources (IUCN) now added this plant in the threatened plant species list (Supe *et al.* 2006). Because of the above mentioned points, it is needed to secure its cultivation for future requirement. Conventional propagation of *W. somnifera* L. primarily via seeds is, however, insufficient to fulfill its demand on large scale because of the poor viability of seeds (Siddique *et al.* 2004) and poor seed germination rates (Vakeswaran and Krishnasamy 2003). Therefore, development of simple and an efficient *in vitro* propagation method via direct somatic embryogenesis may play an important role in rapid multiplication and germplasm conservation of this medicinally important herb.

Formation of bipolar structures from somatic cells has gained attention due to its ability to produce clones that can be used for modifications at genetic level (Sharp *et al.* 1980). Somatic embryos can either be developed directly or indirectly from the explants. Indirect somatic embryogenesis i.e.

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via callus cultures leads to cytological and genetic changes which may not be useful (Larkin and Scowcraft 1981). Direct somatic embryogenesis has better applicability for the micropropagation of plants.

In vitro regeneration of plants from different techniques of tissue culture from a variety of explants using various plant growth regulators has already reported in *W. somnifera* L. (Rani *et al.* 2004, Sivanesan and Murugesan 2008, Logesh *et al.* 2010, Fatima and Anis 2011, Kannan *et al.* 2013, Udayakumar *et al*. 2013, Saema *et al.* 2015, Autade *et al*. 2016) and only few successful reports are available on indirect somatic embryogenesis in *W. somnifera* (Chakraborty *et al.* 2013, Rani *et al.* 2004, Sharma *et al.* 2010, Swathia *et al.* 2013) but till to-date no extensive work was done on direct somatic embryogenesis in *W. somnifera*. Therefore, the objective of the present study was to investigate a simple, efficient and reproducible technique for plantlet formation using nodal explants from direct somatic embryogenesis and to optimize various physio- biochemical activities of acclimatized plants at different time duration.

MATERIALS AND METHODS

Plant material and culture conditions: The nodal explants of *W. somnifera* L. procured from plants grown at the Botanical Garden of Sri Guru Granth Sahib World University, Fatehgarh, were thoroughly washed with tap water for 30 min and then treated with 5% (v/v) teepol for 20 min. The explants were disinfected by immersing in 0.1% (w/v) $HgCl_2$ for 4 min and rinsed 5 times with sterile double distilled water. The nodal explants were cut into standard size (0.5-1.0 cm) and inoculated onto medium containing TDZ. Murashige and Skoog medium (1962) containing 3% (w/v) sucrose was used in all experiments. The medium pH was set to 5.8 before adding agar [0.8% (w/v)] after that media was autoclaved for 20 min at 121 °C and 1.06 kg cm^{-2} pressures. Cultures were placed in culture racks at 24 ± 2 °C with 16 h light/ 8h dark photoperiods (light intensity 50 µmol m^{-2} s^{-1}) illuminated by fluorescent lamps (cool white)with relative humidity approximately 60-65%.

Explant inoculation and formation of somatic embryos: MS medium without thidiazuron was used as control for the whole experiment. For somatic embryos induction, nodal explants were inoculated on MS medium augmented with various concentrations of TDZ (0.8-3.5 µM). All the different stages were recorded in the same TDZ containing MS medium. However, subculturing was done after every week into new MS medium for 3-4 times. Data for percentage and number of somatic embryos per explant was recorded after 3 weeks.

Acclimatization: Plantlets developed from somatic embryos were taken out from the culture vessel then washed with DDW and acclimatized to plastic pots containing sterile soilrite. A transparent plastic bag was put on potted plantlets to maintain high humidity and half strength MS medium was used for watering the plants after every 3 days. Plastic bags were removed for acclimatization. Plantlets were then shifted to pots having normal garden soil and kept in greenhouse under normal environmental conditions.

Histological analysis: For histological analysis, different developmental stages of somatic embryos developed from the upper surface of nodal segments were fixed in FAA [Formalin 5: Glacial acetic acid 5: Alcohol (70%) 90, v/v] for overnight from the best treatment. Fixed explants were dehydrated with ethanol series and then embedded in paraffin wax (melting point 58 ºC). Rotary microtome was used to cut the longitudinal sections of 10 µm thickness. Paraffin ribbons

with sections were pasted on glass slide and were passed through a series of deparaffinizing solutions and stained with Hematoxylin-eosin and then observed under light microscope.

Physiological and biochemical studies: Different physiological parameters i.e. chlorophylls (Chl) a and b, carotenoid contents and net photosynthetic rate ($_PN$), were estimated in plantlets regenerated from somatic embryos. Leaf material was isolated at different time intervals (0, 8, 16, 24 and 32 days) after acclimatization in sterile soilrite. Samples were kept in liquid nitrogen for further studies. $_PN$ was recorded using portable Infra Red Gas Analyzer (IRGA, LI-COR 6400, Lincoln, USA) on fully expanded leaves by placing the leaf in the designated leaf chamber and rate for CO_2 concentration changed over a short time intervals were monitored. Data was recorded on net exchange of CO_2 between leaf and atmosphere and was represented as µmol $CO_2m^{-2}s^{-1}$.

The chlorophyll (chl) a and b and carotenoid contents were analyzed by the procedure described by Mckinney (1941) and McLachlan and Zalik (1963)respectively.

Super oxide dismutase (superoxide: superoxide oxidoreductase, EC 1.15.1.1) activity was measured by the method of Dhinsa *et al.* (1981) with slight changes in concentrations.

Catalase (H_2O_2:H_2O_2 oxidoreductase: EC 1.11.1.6) activity in the leaves of regenerated plantlets was determined by the method of Aebi (1984) with slight changes in concentrations.

The total soluble protein content was assessed following the method of Bradford (1976) using Bovine Serum Albumin (BSA, Sigma, USA) as standard.

Statistical analysis: All the experiments were done for three times and for every concentration, twenty replicates were used. One way analysis of variance (ANOVA) was used for data analysis and the effect of different treatments was determined. Duncan's multiple range test was preferred for means comparison at 5 % significant level.

RESULTS

Induction, germination and maturation of somatic embryos: There was no sign of embryo formation when nodal segments were placed on medium devoid of TDZ. However, when MS medium was augmented with different doses of TDZ (0.8-3.5 µM), proembryos were first observed from the upper surface of the explants and visible after 2 weeks and they were white in colour (Fig. 1a). Development of different stages of somatic embryos i.e. globular, heart, torpedo and cotyledonary stages were observed in the same TDZ containing medium after subculturing (atleast 3-4 times) (Fig. 1b, c, e). The % regeneration of explants forming somatic embryos and subsequent stages of development varied from 40-80% in various concentrations of TDZ. Among a range of TDZ analyzed, best results in terms of induction frequency and number of somatic embryos per explant was recorded on MS medium supplemented with 2.0 µM TDZ as it produced 20.8 ± 1.02 somatic embryos per explant with 80% induction rate (Table 1). Beyond the optimal level, frequency and number of somatic embryos declined gradually when concentration of TDZ was increased. Induction and germination of somatic embryos occurred on same TDZ containing medium. The embryos germinated were attached to the mother tissue, later they were detached from the same(Fig.1f). Greening of cotyledons and radical elongation observed after one week of subculturing, these were the first sign of somatic embryo germination which subsequently turned into plantlets (Fig.1g), eliminated the rooting step and save time and cost.

Table 1: Effect of TDZ on direct somatic embryogenesis from nodal segment of Withania somnifera L. after 3 weeks of culture.

TDZ (µM)	Explant producing somatic embryos (%)	No. of somatic embryos per explant	No. of somatic embryos producing plantlets
0.0	0.0	0.0	0.0
0.8	45	4.5 ± 0.33^{fg}	2.1 ± 0.20^{e}
1.0	60	9.9 ± 0.23^{cd}	4.5 ± 1.05^{cd}
1.5	72	12.7 ± 1.12^{b}	11.7 ± 1.05^{b}
2.0	80	20.8 ± 1.02^{a}	16.2 ± 0.63^{a}
2.5	68	13.2 ± 0.29^{de}	8.3 ± 0.70^{c}
3.0	55	8.6 ± 0.33^{ef}	4.5 ± 0.35^{d}
3.5	40	3.7 ± 0.20^{fg}	1.8 ± 0.34^{ef}

Data represent means ± SE. Means followed by the same letter within columns are not significantly different (P = 0.05) using Duncan's multiple range test

These plantlets were gradually hardened inside the culture room in plastic pots filled with sterile soilrite for 2 weeks, then transferred to greenhouse for a week time and finally shifted to natural conditions for acclimatization.

Histological analysis of somatic embryogenesis: To confirm the formation of somatic embryos from the explant surface, these somatic embryos were subjected to histological examination. Numerous meristematic tissues were observed at the epidermal and subepidermal layers of nodal segment. These cells were small size with dense cytoplasm and obvious nucleus. The meristematic cells differentiated into proembryos followed by the development of globular and heart shaped embryos. Complete cotyledon structure was formed without vascular connection between emerging embryos and the original tissue. These result indicated that the regenerated plantlets were directly developed from the somatic embryos (Fig. 1d).

Physiological and biochemical parameters: The impact of different time duration (0, 8, 16, 24 and 32 days) of transplantation of plantlets formed from the somatic embryos on different physio-biochemical activities viz. analysis of pN, Chl a, b, carotenoid content and antioxidative enzymes (SOD and CAT) were assessed. Chl (a and b), carotenoid content and $_{P}$N was first dropped at day 0 to day 8 then increased from 16-32 day of transplantation (Fig. 2). SOD activity also showed a similar trend but CAT activity was increased throughout all the days of transplantation (0-32) (Fig. 3).

DISCUSSION

The aim of the present study was to develop simple, reliable and economic protocol for direct somatic embryogenesis from nodal segment of *W. somnifera* L. on MS medium supplemented with TDZ. To the best of our knowledge, this is the first report of direct somatic embryogenesis and their conversion into plantlets in the same induction medium. Thidiazuron, a substituted phenyl urea (N-phenyl-1,2,3-thidiazol-5-yl urea) is an effective growth regulator that can be used as cytokinins in various culture systems and more frequently used for induction of multiple shoots (Bukhari *et al.* 2016). TDZ was efficiently used for induction of somatic embryogenesis too in many medicinal plants such as in *Capsicum annumm* (Khan *et al.* 2006) *Paulowania elongata* (Ipekci and Gozukirmizi 2003) and *Nothapodytes foetida* (Khadke and Kuvalekar 2013). In this study, small white globular embryos were conspicuous from the upper surface of nodal

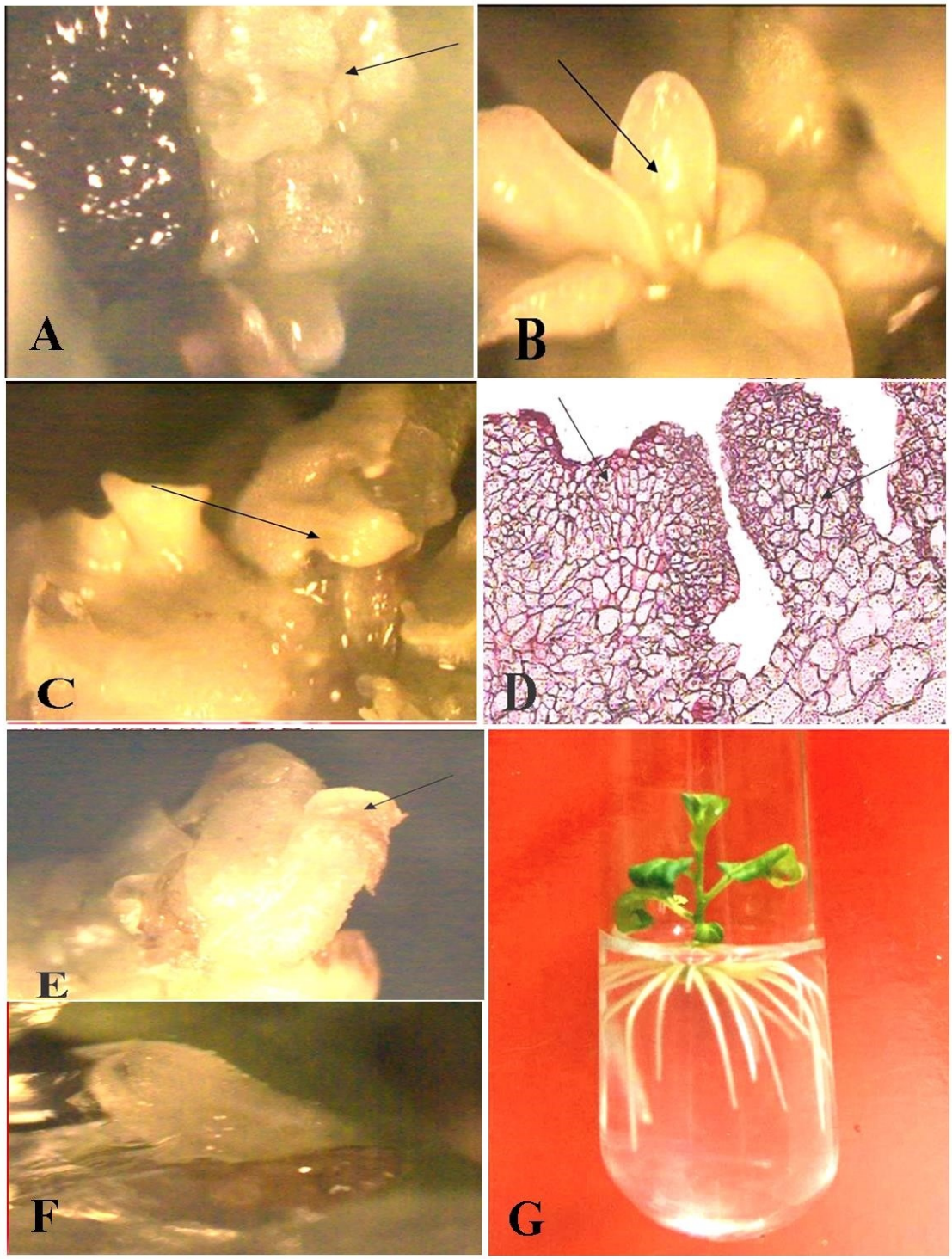

Figure 1:A-G Different stages of development of direct somatic embryogenesis (induction, germination and conversion) in Withania somnifera L. on MS medium +TDZ (2.0 µM) and plantlet formation from somatic embryo after 8 weeks of culture; **A.** Cluster of proembryo attached to the stem explant (arrow); **B.**Globular shaped embryos (arrow); **C.** Heart shaped embryo (arrow); **D.** showing histological analysis; cross section of explant showing asynchronous development of globular and heart shaped embryo from explant (arrow) on MS medium containing TDZ (2.0 µM); **E.** Torpedo shaped embryo; **F.** Germination of embryo; **G,** Rooted plantlet.

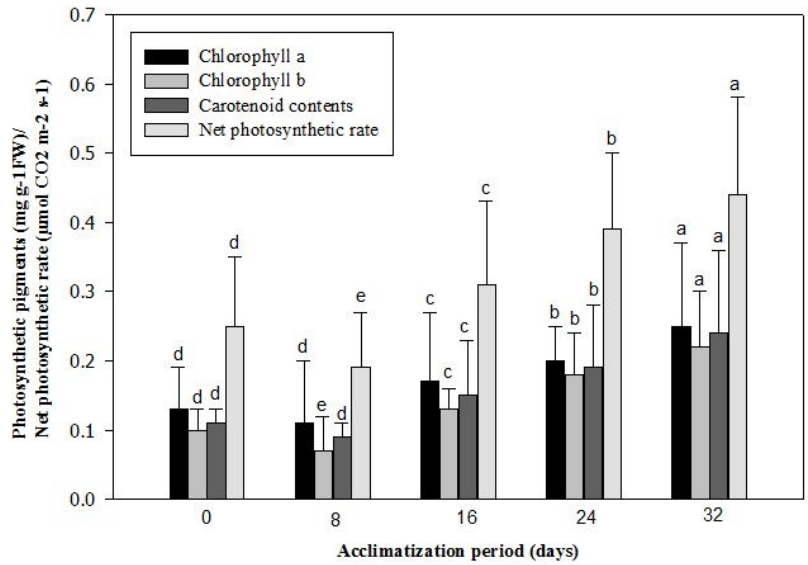

Figure 2: Effect of different days of acclimatization on photosynthetic pigments and net photosynthetic rate ($_{p}$N) of micropropagated plantlets of *W. somnifera*. Bar represents mean ± SE. Bars representing the same letter within response variables are not significantly different (P = 0.05) using Duncan's multiple range test

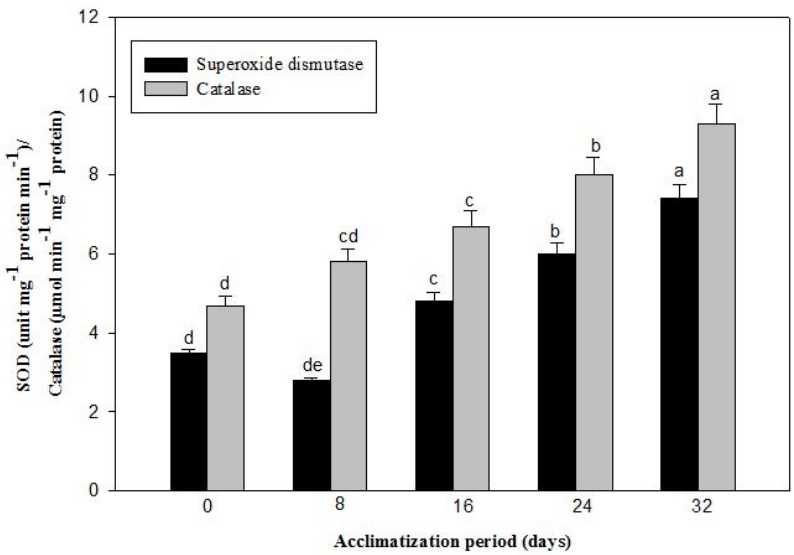

Figure 3: Effect of different days of acclimatization on superoxide dismutase and catalase activity of micropropagated plantlets of *W. somnifera*. Bars represents mean ± SE. Bars representing the same letter within response variables are not significantly different (P = 0.05) using Duncan's multiple range test

segments after one week in culture medium containing different concentrations of TDZ. Concentration of TDZ in medium significantly affected the numbers of embryos per producing explant. TDZ at 2.0 µM showed maximum number of somatic embryos. This was considered the best concentration among all the treatments. The ability of TDZ for direct somatic embryos development at lower concentration has been reported by several workers (Hutteman and Preece 1993, Khan *et al.* 2006, Khadke and Kuvalekar 2013). Globular embryos continued to develop through heart, torpedo and cotyledons stage on the same induction medium containing TDZ. These were bipolar structure flanked by

cotyledons and opaque white in appearance. The formation of somatic embryos was asynchronous. As a result, different stages of somatic embryos development could be observed in cluster of embryos originating from the explants. This was also confirmed by histological analysis. This result was parallel with *Nothapodytes foetida* (Khadke and Kuvalekar 2013) and in *Althea officinalis* (Naz *et al*. 2015). Development of embryos *(*Induction and maturation) involved many steps in a number of plants like sandalwood (Rai and Mc Comb 2002) and *Fagopyrum* (Gumerov*a et al.* 2003) but in the present study, all the developmental stages were recorded in the same medium, provided one step process as in *Capsicum annuum* (Khan *et al.* 2006) and *Nothapodytes foetida* (Khadke and Kuvalekar 2013).

Plantlets regenerated from somatic embryos having 4-5 well formed leaves and roots were acclimatized inside the growth chamber in plastic pots containing soilrite for four weeks and eventually shifted in earthen pots filled with soil. Survival rate of acclimatized plantlets from plastic pots to earthen pots was about 85%. Their growth was normal and reached to maturity in greenhouse.

A number of physiological and biochemical parameters was also analyzed and found that chl (a & b), carotenoid, $_{P}$N and SOD was first declined then after significantly increased whereas CAT activity was enhanced in all days tested. This could be due to change in environmental conditions from culture room to natural conditions and better suitability of plantlets to overcome the stress generated from reactive oxygen species (ROS). Simliar results were also noticed in *Cassia angustifolia* (Bukhari *et al.* 2014), *Rauvolfia tetraphylla* (Faisal and Anis 2009) and *Capparis decidua* (Siddique and Bukhari 2018).

CONCLUSION

To conclude the present experiment, it describes a simple and rapid protocol for large scale micropropagation of *W. somnifera* L. via direct somatic embryogenesis. Physio-biochemical study suggested better adaptation of regenerated plantlets to natural environment. The protocol developed here would also helpful in genetic transformation and for the conservation of germplasm.

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