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IN-VITRO GERMINATION AND SOMATIC EMBRYOGENESIS *VIA* TISSUE CULTURE STUDIES OF *TAVERNIERA CUNEIFOLIA* (ROTH) ARN.

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ABSTRACT: *Taverniera cuneifolia* (Roth) Arn. licorice of India comprises similar phytochemical constituents majorly the Glycyrrhizic acid (GA) that of plant *Glycyrrhiza glabra* L. but has been least studied in terms of their commercial applications. GA from the root extracts of *T. cuneifolia* exhibits promising anti-inflammatory, anti-tumor, and anti-germ tube formation protection and other cytotoxic activities. To examine the same, present study experiments on methods for 1. Development of somatic embryo from root culture 2. To regenerate plants using root cultures. The experimental results showed the shoot initiation and increased rate of root growth, ranging from 65.4% to 89.3%, while plant regeneration from somatic embryos was 82.6% when cultures in ¼th strength Murashige and Skoog (MS) medium with 2% sucrose as a supplement. The study results in proliferate germination of seeds in respective media and suggests that *T. cuneifolia* could be used as a substitute of *G. glabra* in the practice of efficient mass production of plants from a single mother plant.

INTRODUCTION: Secondary metabolites in plants are vital phytoconstituents for their application in pharmaceutical, food, and chemical industries. Ancient India records the application of herbs and medicinal plants to cure effortlessly, the primary diseases such as intestinal infections, inflammations, anti-bacterial/fungal, and most topical treatment of skin and wounds [12]. The 21st-century pharmacognosist route the same path as of ancient science followed; to uncover the new biomolecules from plant extracts of medicinal properties and apply against todays drug resistant bacteria and fungi.

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This method of using traditional plants circumvents loss of native species and aid in quantitative extraction and comparison of extracts of plants with similar biomolecules. Amongst several medicinal plants across the globe, one such uniqueness is the plant Taverniera and its extract glycyrrhizic acid (GA). Genus Taverniera records the medicinally important triterpene glycoside found in similar leguminous plants like *Glucurrihiza glarba* L. (Commonly known as licorice) and *Abrus precatorius* L. (precatory bean) [2]. This compound in active form serves as an anti inflammatory, antiallergic, anticancer and immune-modulating activities [10] well known to inhibit HIV, hence, practiced in treating HIV patients while the mono ammonium salt (glycyrram) is used as a sweetener and is a taste improving nutrient additive in the food industry. This compound in replacement to prednisolone is also implemented as a treatment of bronchial asthma in children [5].

Licorice, the roots of the *Glycyrrhiza* plant species, has been used for more than 4000 years, with approximately 30 species, of which six species produce a sweet saponin glycyrrhizic acid (GA) (16% GA), and they are widely used in Asia countries, imports from Pakistan and Afghanistan [1]. The genus Taverniera (Indian licorice) grows in bushes land limestone with an altitude range of 1700 to 2300 above sea level. Being a threatened plant, Taverniera of family Fabaceae includes 12 species, recognized as endemic to parts of Africa and Southern Asian countries. As of plant extracts belonging to Glycyrrhiza species, 13% of glycyrrhizic acid is extracted from the roots of *Taverniera cuneifolia* proving its vital role in producing GA however, less likely explored but used as a substitute for *G. glabra*. Although studies prove the use of Taverniera roots in medicines against bacterial and fungal infections, but not as much known about its propagation, regeneration, and the phytoconstituents are available [6]. Owing to the demand in the industries, Taverniera plant targeting Glycyrrhizic acid (GA) is mandatorily in need for continued industrial production of medicines.

Prior to studying the application of phytochemical constituents of Taverniera, it is essential to understand the methods of plant regeneration characteristics such as tissue culture techniques *i.e.*, callus culture, suspension and root cultures, and other different growth strategies. The *in-vitro* root culture provides a rapid and continuous source of biomass, whereas somatic embryogenesis through micropropagation, genetic transfer, and synthetic seed preparation plays a vital role in developing plants in large biomass, thus potential on a commercial scale. To attain a large number of uniform pest / disease-free plants, *in-vitro* regeneration developed by somatic embryogenesis is an apt method amongst all. Further, methods need clarification on the mass propagation, physiological, morphological, and molecular studies and for the conservation of desired genotypes for the regeneration model of somatic embryogenesis. Somatic embryos from root explants is known to be reported in trees and few herbaceous plants; thus, in the present study, we aim to develop an effective method for regeneration through somatic embryogenesis and complete formation of plant *Taverniera cuneifolia*.

The study also experiments and demonstrates the embryonic cell suspension culture from root explants and establishes the same without the use of external growth regulators.

MATERIALS AND METHODS:

Media and Reagents: Murashige and Skoog (MS) media were purchased from Hi-Media Laboratories Pvt. Ltd. (Mumbai, India). $HgCl_2$ and other reagents purchased from Merck Laboratories (USA)

Collection and Maintenance in Aseptic Conditions: *T. cuneifolia* plants were collected from the Omarabad district of Maharashtra and identified with the help of a standard identification book on local flora. Herbarium of a voucher specimen (voucher number 921) was deposited to Medicinal Plants Conservation Center, Pune, and Maharashtra State, India. The seeds from the identified plant were collected and surface sterilized in a laminar airflow chamber using detergent wash followed by a wash with 70% ethanol for 3 min, followed by a wash with 0.1% $HgCl_2$ for 1 min.

Samples were repeatedly rinsed four times with sterile distilled water. Various culture media such as Murashige and Skoog (MS), 1/2-strength MS and 1/4-strength MS fortified with varying concentrations of sucrose (1%, 2%, and 3%) were used for seed germination. pH was adjusted to 5.8 ± 0.2 before sterilization at 120 °C and at 15 lb pressure and inoculated in glass tubes. A set of seeds was ruptured using a surgical scalpel blade (No. 10) and inoculated in glass tubes (22 mm × 150 mm) containing 25 ml of semi-solid MS medium. The seeds were maintained at 25 ± 2 °C, 16 / 8 h photoperiod using cool white fluorescent light.

Root Cultures and Induction of Shoots: Roots were grown *in-vitro*; once germinated (~2 weeks) the seedlings were transferred and inoculated in Petri-plates containing various strengths of semi-solid MS medium fortified with various concentrations 3w of sucrose. ~10-15 root segments of 2-3 cm were inoculated on 20 ml of semisolid MS medium, incubated 16/8 h photoperiod at 25 ± 2 °C, and subcultured every three weeks.

Once the shoots grow, the subculturing was repeated using fresh MS medium to generate complete plants. 0.1 g root was weighed and inoculated in a 100 ml flask containing with 50 ml of MS basal liquid medium + various strengths (full MS, 1/2 MS, and 1/4 MS), and varied with concentrations of sucrose (1%, 2%, and 3%) and incubated at 25 ± 2 °C on a rotary shaker incubator at 80 rpm. Each concentration was maintained with minimum of 6 replicates by washing with a new medium every 4 weeks.

Induction of Somatic Embryos and Plant Regeneration: The excised roots of the germinated plants were maintained in the induction liquid (1/4 strength MS fortified with 1% sucrose) medium for inducing somatic embryos. Once the embryogenic suspension initiates from root cultures, it is transferred to the Petri-plates containing various strengths of MS (full MS, 1/2 MS and 1/4 MS) medium / different concentrations of sucrose (1%, 2%, and 3%) maintained under 16 / 8 h photoperiod at 25 ± 2 °C. Thus, newly developed plants were sub-cultured and maintained on 1/4 MS medium supplemented with 100 ml/L of coconut water for further, development. Regenerated shoots and well-developed plants from somatic embryos were washed thoroughly in running water and transferred to plastic pots containing sterile coco peat, moistened by 1/8 MS basal salt solution with 100 mg l-1 Bavistin ® (BVN carbendazim powder, BASF, Mumbai, India) for preventing fungal growth. This is then covered with transparent polythene bags for two weeks to prevent desiccation. It takes 3 weeks to remove plants from coco peat and planted individually in pots and maintained in shades (containing a garden soil/vermiculate/ sand mixture at 2:1:1 ratio at 150 g/pot).

RESULT: The main aim of the research work was to standardize the method for the germination and somatic embryogenesis of the selected plant. First, aseptic seed germination of *T. cuneifolia* on different media was performed and compared for their growth. For the comparison of the germination rate, half of the seeds were ruptured and another half were incubated without rupturing and maintained in the laminar flow **Fig. 1a, b.** The ruptured seeds showed swelling within 3 days, roots visibility within 5 days, whereas unruptured seeds took 13-15 days for germination **Fig. 1c.** The percentage or rate of germination of seeds inclined by 15% in ruptured seeds and are represented in **Table 1.**

TABLE 1: GERMINATION FREQUENCY OF SEEDS OF *TAVERNIERA CUNEIFOLIA*, IN RUPTURED AND INTACT SEEDS UNDER ASEPTIC CONDITIONS

MS media		Germination frequency	
Strength	Sucrose (%)	Ruptured seeds (%)	Intact seeds (%)
One-Fourth	1	93	81
	2	96	80
	3	94	83
Half	1	95	86
	2	97	88
	3	99	87
Full	1	97	88
	2	99	87
	3	100	91

Fig. 1d represents the seed germination and formation under aseptic conditions, initial root appearance on semi-solid medium, and swollen ruptured roots. The maximum germination of 96% in ruptured and 85% growth in intact seed cultures was recorded in MS fortified full-strength media **Fig. 1e.**

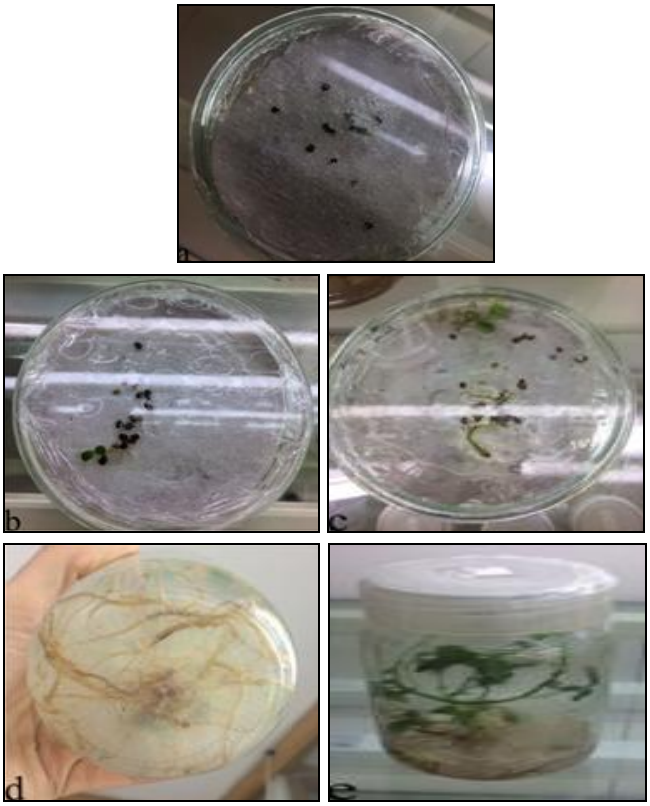

FIG. 1: A, B. INITIAL GERMINATION OF UNRUPTURED (A) AND RUPTURED SEEDS (B); C. INITIATION OF GROWTH OF SHOOTS AND ROOTS; D. PROLIFIC GROWTH OF ROOTS; E. COMPLETE GROWTH OF PLANT

The post-germination period was examined for the growth of root cultures and recorded through photographs and measurements. The 0.1 g root pieces were grown into full root system after the second week of seed germination in different strengths, with 1-3% sucrose. The swelling of roots was observed only after 12 days of longitudinal root growth, which later became whitish in color in the next 15 days' time period, thus forming the callus-like mass formation **Fig. 2a, b.** Further, shoot initiated post 30 days of culturing **Fig. 2c, d,** and shoot buds appeared in all plates ranged 60 92% with the highest number of shoots in all the media only in 3% sucrose. The percent of sucrose, the strength of the media, and shoot initiation frequency is mentioned in **Table 2.**

TABLE 2: EFFECT OF DIFFERENT STRENGTHS OF MS MEDIA AND SUCROSE CONCENTRATIONS ON SHOOT INITIATION FREQUENCY IN CULTURES ROOT OF *T. CUNEIFOLIA*

MS medium		% Shoot initiation frequency (± SD)
Strength	Sucrose (%)	
One - fourth	1	63.3 ± 0.66
	2	59.2 ± 0.31
	3	61.5 ± 1.1
Half	1	65.01 ± 0.76
	2	70.62 ± 0.32
	3	69.39 ± 1.44
Full	1	82.44 ± 1.77
	2	86.02 ± 0.99
	3	91.00 ± 0.16

The shoot formation appeared, which was then transferred to new flasks, and thus, the whole plant was obtained after 15 days of the root formation **Fig. 2c, d.**

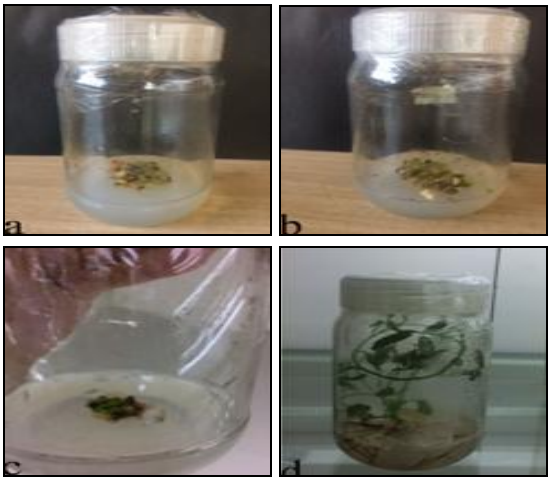

FIG. 2: A, B. PHOTOGRAPH OF CALLUS; C. PHOTOGRAPHS OF INITIATION OF SHOOT BUD FROM THE CALLUS; D. COMPLETE GROWTH OF PLANT FROM THE CALLUS IN MS MEDIUM

Embryo Formation: Embryo formation took approximately three months from the time of shoot initiation. As the roots released viable cells, media turned turbid due to various stages of somatic embryos, suspended cells, and roots. The germination of embryos initiated by transferring a few drops of embryonic suspension ion to petri plate containing media with varying sucrose concentrations 1%, 2%, and 3%; **Fig. 3.** Green mat-like growth on the petri plate took 15 days, while callus like mass embedded on green mat took another 15 days during embryo formation. It took more than 85% regeneration time for the somatic embryo formation on 1/4 -strength MS medium supplemented with 2% sucrose **Table 3.** If the callus formation was more profound, leading the secondary embryogenesis, the somatic embryos were sub-cultured to 1/4-strength of MS semisolid medium fortified with 2% sucrose. Subsequent to regeneration, an entire plant from somatic embryos was transferred for hardening and later transferred to the field.

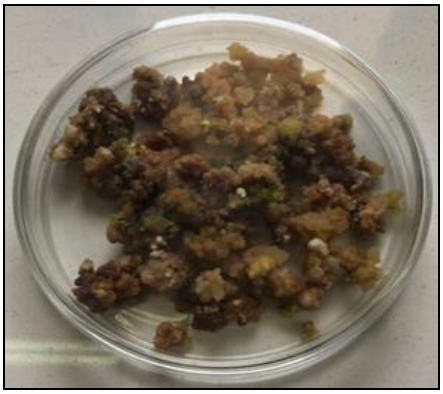

FIG. 3: PHOTOGRAPH OF CALLUS IN BROWN COLOUR, GREEN BUD INITIATION IN BETWEEN THE CALLUS IS ALSO OBSERVED

TABLE 3: EFFECT OF STRENGTHS OF MS MEDIA AND SUCROSE CONCENTRATION ON PLANT REGENERATION FROM SOMATIC EMBRYOS IN *T. CUNEIFOLIA*

MS medium		% Frequency of plantlet regeneration from somatic embryos (± SD)
Strength	Sucrose (%)	
One -fourth	1	79.02 ± 0.33
	2	87.2 ± 0.97
	3	76.11 ± 0.16
Half	1	69.23 ± 1.03
	2	71.0 ± 0.2
	3	66.33 ± 1.45
Full	1	77.5 ± 0.89
	2	82 ± 1.1
	3	80.9 ± 1.56

DISCUSSION: Plant tissue culture is well known for initiating plant tissues and growing them on nutrient media, where the type of plant depends on the factors such as propagation, seeds variety, and variation in media. In this study, we demonstrate the establishment of root cultures, shoot growth, regeneration of complete plant from the root sin culture media, and somatic embryogenesis from the cultured roots of the plant *T. cuneifolia.* First, the plant growth from ruptured and non-ruptured seeds was examined prior to the selection of seeds for embryogenesis.

During this process, ruptured cell results in fast germination of seeds and induction of roots and shoot, and the increase in the level of sucrose in the media leading to increased shoot length was recorded. Sucrose in the MS medium acted as a fuel source for sustained metabolism, ensuring optimal development in cases like poorly developed cellular and tissues, lack of chlorophyll, *etc.* The level of sucrose also supports the maintenance of osmotic potential and conservation of water in cells [3]. The regeneration of entire plant from the root in tissue culture has been reported earlier [1] the other studies proves that the root and shoot development from the ruptured cell could be due to the plasticity of post-embryonic development in plants, which sustains the ability to re-generate whole new organs such as roots, leaves or flowers, during life cycle [9].

The well-developed callus from the plants is also obtained through two vital components, phytohormones- cytokine and auxin, and their ratio in culture media [11]. While this study holds the observations on the next type of regeneration where peculiar somatic embryogenesis occurs when true embryos are formed from somatic cells either *in-situ* or *in vitro.* In the present study, the use of semi-solid MS Medium for the profuse shooting of the plant (nearly 100% after hardening) is the highlight to explain the importance of the choice of medium for tissue culture. On the contrary, in liquid media, roots were suspended as cells that formed globular embryo-like structures of up to 2 mm, brownish to white color with a smooth rounded appearance. This culture appears as a turbid mixture of roots, cells, cells aggregates, and embryos. Now, the embryogenesis and formation of the plant from callus depend on several factors, use of media, % sucrose, supplementary and the plants itself are few of those which can be listed through previous studies [15] records 100% shoot regeneration in 5% sucrose supplemented with 4mg/L NAA+ 4mg/L TDZ and embryogenesis in the medium containing 2mg/L IAA + 2mg/L TDZ and 100mg/L ABA which yielded the maximum number of somatic embryos in *Crocus oliveri.* The use of different explants also plays a major role in the regeneration and embryogenesis of the plant along with the proportionate addition of auxins (NAA / IAA) and cytokinins (BAP / TDZ) [14].

For example, Abscisic acid (ABA) is known to impact in exogenous applications of the hormone initiation during rapid cell proliferation, cell expansion, and differentiation, effects on the cell cycle regulation and cell division [4]. It is thus concluded that cultures in the full strength of MS medium tended to induce profuse callus growth while 1/4 -strength MS showed a higher frequency of germination and therefore translated into more yield somatic embryos and successfully yield of hardening plants. However, there was no supplement of auxins and cytokinins provided during the current study, yet; authors could achieve maximum growth of callus and plant regeneration in MS Medium-full strength in Hormone independent study.

At times the plant exposed to natural bacterial infection such as agrobacterium, rhizogenes enabling the culture with its own auxin supplement, as said in Awad *et al*., 2011 [1]. For the commercial application, authentication of plant and systematic characterization are mandatory to continue the use of these plants and their application in industrial pilot study experiments. Sustainable exploitation of plants for medicinal value and conservation of invaluable resources are the major concern in today's research development. The location of plant collection, authentic characteristic details, and report of herbarium to Medicinal plant conservation center would help other researchers trial the same and explore extended studies on a similar plant. In this study, root cultures are used for the development of the new plant, which is considered as a useful alternative to clonal propagation and germplasm conservation [7, 8;] however, slower growth in the current research could be a downside of the study.

The culture also highlighted the scope in the extraction of glycyrrhizic acid (GA) from *Taverniera cuneifolia* through the HPTLC method and compared it with the GA reference standard.

CONCLUSION: For any tissue culture studies, the methods of shoot initiation and germination is vital for the complete callus formation and development of embryos. This research study concludes that the somatic embryogenesis of *Taverniera cuneifolia*, which was achieved within 15 days; however, the ruptured seeds shown a high germination rate compare to unruptured seeds. The main outcome was the use of different concentrations of sucrose (1%, 2%, and 3%), which exhibit the green mass growth and patches of undifferentiated callus-like mass embedded within developing embryos.

Further, the study results are implemented and continued for quantification of glycyrrhizic acid (GA) by an analytical method and are submitted elsewhere. From this study, we can develop plant tissue culture at the specific and aseptic conditions at laboratories and scale to pilot, and same applied for industrial extraction of glycyrrhizic acid.

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CONFLICTS OF INTEREST: Nil

REFERENCES:

1. Awad V, Shirke R, Mukherjee S, Khadke S, Pawar P, Meti N and Harsulkar A: Somatic embryogenesis, regeneration and *in-vitro* production of glycyrrhizic acid from the root culture of it a *Verniera cuneifolia* (Roth*)* Arn. *In-vitro* Cellular and Development Biology- Plant 2011; 47(4): 525-35.
2. Baltina LA: Chemical modification of glycyrrhizic acid as a route to new bioactive 2003;
3. Bhatia S, Sharma K, Dahiya RS and Bera T: Modern applications of plant biotechnology in pharmaceutical sciences. Academic Press Compounds for Medicine Current Medicinal Chemistry 2015; 10: 155-71.
4. Francis D and Sorrell DA: The interface between the cell cycle and plant growth regulators: A mini review Plant Growth Regulation 2001; 33(1): 1-12.
5. Kondratenko RM, Baltina L, Mikhailova LR, Danilov VT, Gabbasov TM, Murinov YI and Tolstikov GA: Obtaining glycyrrhizic acid and its practically useful salts from a commercial licorice root extract. Pharmaceutical Chemistry Journal 2005; 39(2): 84-88.
6. Mangalorkar P, Patel M, Shah, A and Nagar P: Chemical profiling of *Taverniera cuneifolia* (Roth) Ali, a possible substitute of *Glycyrrhiza glabra* L. by GC-MS. World Journal of Pharmaceutical Research 2016; 5(2): 547 -52.
7. Mathur M, Yadav S, Katariya PK and Kamal R: *In-vitro* propagation and bio-synthesis of steroidal sapogenins from various morphogenetic stages of *Moringa oleifera* Lam. and their antioxidant potential. Acta Physiol Plant 2013; DOI 10.1007/s11738-014-1549-1.
8. Rajasekharan PE and Sahijram L: *In-vitro* conservation of plant germplasm. Eds B Bahadur *et al.,* Plant Biology and Biotechnology Volume II: Plant Genomics and Biotechnology 2015; DOI 10.1007/978-81-322-2283-5-22.
9. Sena G: Stem cells and regeneration in plants. Nephron experimental nephrology. Nephron Experimental Nephrology 2014; 126: 35-39.
10. Singh A and Navneet: A review on medicinal plants and herbs of Uttrakhand India: its traditional, ethnomedicinal and antimicrobial potential. Nature and Science 2016; 14(12): 90-07.
11. Skoog F and Miller C: Chemical regulation of growth and organ formation in plant tissue cultured *in-vitro*. Symp Soc Exp Biol 1957; 111(1): 118-31.
12. Tambekar DH and Dahikar SB: Antibacterial activity of some Indian Ayurvedic preparations against enteric bacterial pathogens. Journal of Advanced Pharmaceutical Technology and Research 2011; 2(1): 24-29.
13. Tian M, Yan H and Row KH: Extraction of glycyrrhizic acid and glabridin from licorice. International journal of Molecular Science 2008; 9: 571-77.
14. Vatankhah E, Niknam V and Ebrahimzadeh H: Activity of antioxidant enzyme during *in-vitro* organogenesis in crocus sativus. Biologia Plantarum 2010; 54(3): 509-14.
15. Verma SK, Das AK, Cingoz GS, Uslu E and Gurel E: Influence of nutrient media on callus induction, somatic embryogenesis and plant regeneration in selected Turkish crocus species. Biotechnology Reports 2016; 10: 66-74.

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