Glycyrrhizic acid (GA) from *Taverniera cuneifolia* (Roth) Arn.: In vitro germination and somatic embryogenesis via tissue culture studies

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**ABSTRACT**

Glycyrrhizic acid (GA) is commercially extracted from plant *Glycyrrhiza glabra* L., while *Taverniera cuneifolia* (Roth) Arn, licorice of India comprise similar phytochemical constituents but has been least studies on commercial applications. In 21st century, scientist and pharmacognocist explored new biomolecules in several plants which proved to comprise medicinal properties for examination against bacteria and fungi, to circumvent loss of native plants, for better and quantitative extraction using plants of similar properties. GA from the root extracts of plants exhibits promising anti-inflammatory, anti-tumour and anti-germ tube formation protection and other cytotoxic activities. This compound has pharmacological properties such as HIV inhibition, in the food sector, mono-ammonium salt (glycyrram) is a form of sweetener with nutrient enhancement properties. The present study experiments on methods for Development of somatic embryos from root culture and to regenerate plant using root cultures. The observations revealed that, shoot initiation and rate of root growth ranged from 65.4% to 89.3% while plant regeneration from somatic embryos was 82.6% when cultures in 1/4th strength Murashige and Skoog (MS) medium by sucrose 2% used as a supplement. The study illustrates that proliferative germination of seeds in respective media and also suggested that *T. cuneifolia* could be used as substitute of *G. Glabra* used as an systematic methodology and plants are mass-produced from a single mother plant.

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**INTRODUCTION**

Secondary metabolites in plants are vital phyto-constituents for their application in pharmaceutical, food and industries. Ancient India records use of herbal medicinal plants for the therapy of primary diseases of intestinal infections, inflammations, anti-bacterial/fungal or mostly topical applications of medicines which were cured effortlessly (*Tambekar and Dahikar, 2011*). The study and extraction of Glycyrrhizic acid (GA) from the genus *Taverniera* is one such rarity. Glycyrrhizic acid extraction (GA) like a triterpene glycoside found in leguminous plants like *Glucurrihiza glabra* L. (Commonly known as licorice) and *Abrus catorius* L. (precatory bean) (*Baltina, 2003*). This compound inactive form responds as anti-inflammatory, antiallergic, anticancer and immunomodulating activities (*Singh and Navneet, 2016*). This compound has pharmacological properties such as HIV inhibition, in the food sector, mono-ammonium salt (glycyrram) is a form of sweetener with nutrient
enhancement properties. Prednisolone’s activity in the treatment of bronchial asthma in children has been restored (Kondratenko et al., 2005).

Owing to the demand in the industries, plants containing Glycyrrhizic acid (GA) is mandatorily in need for continued industrial production of medicines. Licorice, which is made from the roots of Glycyrrhiza plant species, has been used for over 4000 years. There are about 30 species, six of which yield the delicious saponin glycyrrhizic acid (GA) (16 percent GA), and they are commonly used in Asia countries (Tian et al., 2008), mainly imported from Pakistan and Afghanistan (Awad et al., 2011).

On contrary to the use of Glycyrrhiza species, the roots of Taverniera cuneifolia, contains 13% of glycyrrhizic acid (GA), equally potent in producing GA is less likely explored but used as substitute for G. glabra. Taverniera belongs to the Fabaceae family and has 12 species. It is an Indian licorice that is widely distributed in Africa and Southern Asia. This is a vulnerable medicinal plant that grows in a limestone bushland area between 1700 and 2300 meters above sea level. Species Taverniera cuneifolia exhibits various properties like expectorant, blood purification, wound healing and as well as in treating spleen tumours (ref). 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 (Mangalorkar et al., 2016).

Prior to understand the phytochemical constituents, it is essential to examine the regeneration characteristics of the plant to proclaim the plant for industrial applications. Recently, the tissue culture technique i.e. callus culture, suspension and root cultures are different strategies, amongst which in vitro root culture provides rapid and continuous source of biomass to industries. Somatic embryogenesis through micropropagation, genetic transfer, and synthetic seed preparation and so on plays a vital role in developing plants in large biomass, thus potential on commercial scale. The in-vitro regeneration developed by somatic embryogenesis will quickly produce large numbers of homogenous, pest- and disease-free plants for testing the efficacy of therapeutic components. The studies on mass propagation, physiological, morphological and molecular studies and for the conservation of desired genotypes, what best suits are the regeneration model of somatic embryogenesis.

Somatic embryos in the root explants to be report in trees and some herbaceous plants. In the present study, we set two objectives, 1. To develop effective method for regeneration through somatic embryogenesis and complete formation of plant Taverniera cuneifolia. 2. To isolate and quantify GA from roots of Taverniera cuneifolia. We have performed and demonstrated embryonic cell suspension culture was obtained from root explants, and it was established without the need of any external growth regulators.

**MATERIALS AND METHODS**

**Collection and maintenance in aseptic conditions**

*T. cuneifolia* plants were collected in Maharashtra’s Osmanabad area and analysed as per the customized identification book on local flora. The Medicinal Plants Conservation Center in Pune, Maharashtra, India, received a voucher specimen of recognized plants specimen (voucher number 870). The seeds which were collected from the plants were processed for the experimental setup. Sequence wise seeds were sterilized and wash with the detergent carried out with 70% ethanol in the laminar air flow chamber for 3 minutes, after that cleanse with 0.1% HgCl₂ up to one minute then replicate fourfold by rinsing distilled water that is sterile. Murashige and Skoog (MS), 1/2-strength MS, and 1/4th-strength MS supplemented with different grades of sucrose concentrations (1 percent, 2 percent, and 3 percent) were used to seed germination at pH 5.80.2 before being sterilized at 120°C and 15 pound pressure. Using a surgical scalpel blade, the respected set of surface sterilized seeds was ruptured (No.10). Both split and undamaged seeds were infected in glass tubes (22 mm150 mm) containing 25 ml of semi-solid MS medium including agar (0.8 percent) as a gelling agent, and the seeds were kept at 252°C for 16/8 hours under cool white fluorescent light. The work’s overall pipeline is represented in Figure 1 of Awad et al. (2011).

**Root cultures and shoot induction:**

In vitro procedures were used to extract roots and seeds, which were then germinated for 2 weeks before being infected in Petri-plates consisting varied quantities of semi-solid MS medium enriched with different grades of sucrose. A total of 0.1 g of root tissue with 10–15 or 2–3 cm root segments was inoculated on 20 ml of semisolid MS medium and incubated at 25±2°C under a 16/8 photoperiod. Every three weeks, sub-culturing was performed. The induced shoots were removed from the roots and subculture in new MS media to produce entire plants. Two weeks following seed germination, roots were removed from the stem. In a 100 mL flask, mix 50 mL MS baseline liquid medium with dif-
ferent strengths (full MS, 1/2 MS, and 1/4 MS) fortified with varying quantities of sucrose, pre-weighed (0.1 g) excised roots were infected (1 percent, 2 percent, and 3 percent). The flasks were incubated for a period of time at 25±2°C for 80 rpm on a rotary shaker incubator. Every four weeks, the medium was replaced, with a minimum of six replicates for each treatment.

**Plant regeneration and induction of somatic embryos**

The germinated plants’ excised roots were kept in an induction liquid (1/4-strength MS supplemented with 1% sucrose) medium to induce somatic embryos. 1 mL of embryogenic solution was placed in Petri-plates with varying MS strengths (full MS, 1/2 MS, and 1/4 MS) medium enriched with varied quantities of sucrose (1 percent, 2 percent, and 3 percent), and kept at 25±2°C under a 16/8 h photoperiod. For continued development, newly produced plants were sub-cultured and maintained on 1/4 MS medium supplemented with 100 ml/L coconut water.

Well-developed plants from somatic embryos were selected and regenerated shoots from root explants cut from the semi-solid medium, and thoroughly washed in running water. The shoots were placed in plastic pots with sterile coco peat, watered with a 1/8 MS basal salt solution containing 100 mg l-1 Bavistin® (BVN carnbandazim powder, BASF, Mumbai, India) to avert fungal development, and covered with transparent polythene bags for two weeks to stop desiccation. Plants were taken from coco peat after three weeks and planted separately in pots (containing a 2:1:1 ratio of garden soil, vermiculate, and sand at 150 g/pot). For three weeks, all of the planted pots were kept in the shade.

**RESULTS**

*T. cuneifolia* seed germination was executed aseptically on several media, consisting full-strength, 1/2-strength, and 1/4-strength MS basal medium. For germination rate comparison, half of the seeds were punctured with a sterile scalpel blade (no. 10) while the other half were incubated without rupture and retained in the laminar flow (Figure 1a, b). The ruptured seeds swelled within 3 days with visible emergence of root within 5 days, whereas enrap-tured seeds took 13-15 days for germination (Figure 1c). The percentage or rate of germination of seeds inclined by 15% in ruptured seeds and are represented in Table 1. The seed germination, formation of plants under aseptic conditions, Initial root appearance on semi-solid medium and swollen roots are represented in Figure 1d. Amongst all the media tested, MS fortified full strength media with 3% sucrose showed maximum germination of 96% in ruptured cell and 85% growth in intact seed cultures (Figure 1e).

Post germination period was examined for the growth of root cultures and recorded through photographs and measurements. After the second week of germination, root cultures were initiated by inoculating 0.1 g of excised root sections with MS of various intensities (as mentioned in materials & methods) with 1–3% sucrose. The swelling of roots was observed only after 12 days of longitudinal root growth, which later became whitish in colour in next 15 days’ time period. Thus, the appearance of a callus-like mass of undifferentiated cells were shaped (Figure 2a, b). Further, shoot initiation took place only after 30 days of culturing in same strength of culture (Figure 2c, d). The rate of growth of shoot buds appeared in all the plates ranged 60-92% with highest number of shoots in all the media only when it was fortified with 3% sucrose. The percent of sucrose, strength of the media and shoot initiation frequency is mentioned in Table 2. In order to avoid root browning due to excessive growth, cultures were sub-cultured every three weeks.

The plant, together with the roots, were refilled with semi-solid material in new flasks of relevant strength and concentration during the initial shoot creation stage, and the entire plant was acquired after 15 days of the process (Figure 2c, d). These plants were acclimatized at a temperature of 25°C, in a poly-house with sterile coco peat.

**Embryo formation**

The formation of embryos acquired approx. three months after inoculation and incubation in rotary shaker. The fluid became murky as the roots released viable cells, reflecting various stages of somatic embryos, suspended cells, and roots. Embryo germination was started by transferring a few drops of embryonic suspension ion to a petri dish containing media with different sucrose concentrations (1 percent, 2 percent, and 3 percent; Figure 3). Green growth was visible on the plate after 15 days, within growing embryos, regions of undifferentiated callus-like material which began to grow in the next 15 days. On 1/4-strength MS medium supplemented with 2% sucrose, somatic embryo development took more than 85 percent of the regeneration period (Table 3).

If secondary embryogenesis developed, i.e., a more thorough induction of callus, the somatic embryos were sub-cultured to 1/4-strength MS semisolid medium enriched with 2% sucrose under any condition. All plants were transplanted to fresh medium
Table 1: Germination frequency of seeds of *Taverniera cuneifolia*, in ruptured and intact seeds under aseptic conditions

<table>
<thead>
<tr>
<th>MS Media Strength</th>
<th>Sucrose (%)</th>
<th>Germination Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ruptured Seeds (%)</td>
</tr>
<tr>
<td>One-Fourth</td>
<td>1</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>95</td>
</tr>
<tr>
<td>Half</td>
<td>2</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>97</td>
</tr>
<tr>
<td>Full</td>
<td>2</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 1: (a). Initial germination of unruptured; (b). ruptured seeds (c). Initiation of growth of shoots and roots; (d). Prolific growth of roots; (e). Complete growth of plant
Figure 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

Table 2: Effect of different strengths of MS media and sucrose concentrations on shoot initiation frequency in cultures root of *T. cuneifolia*

<table>
<thead>
<tr>
<th>Strength</th>
<th>MS Medium</th>
<th>Sucrose (%)</th>
<th>% Shoot initiation frequency (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>One -Fourth</td>
<td>1</td>
<td>63.3±0.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>59.2±0.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>61.5±1.1</td>
<td></td>
</tr>
<tr>
<td>Half</td>
<td>1</td>
<td>65.01±0.76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>70.62±0.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>69.39±1.44</td>
<td></td>
</tr>
<tr>
<td>Full</td>
<td>1</td>
<td>82.44±1.77</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>86.02±0.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>91.00±0.16</td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Effects of MS media strengths and sucrose content on *T. cuneifolia* plant regeneration from somatic embryos

<table>
<thead>
<tr>
<th>Strength</th>
<th>MS Medium</th>
<th>Sucrose (%)</th>
<th>% frequency distribution in of plantlet regeneration carried out in somatic embryos (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>One -Fourth</td>
<td>1</td>
<td>1</td>
<td>79.02±0.33</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>87.2±0.97</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3</td>
<td>76.11±0.16</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>69.23±1.03</td>
</tr>
<tr>
<td>Half</td>
<td>2</td>
<td>2</td>
<td>71.0±0.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3</td>
<td>66.33±1.45</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>77.5±0.89</td>
</tr>
<tr>
<td>Full</td>
<td>2</td>
<td>2</td>
<td>82±1.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3</td>
<td>80.9±1.56</td>
</tr>
</tbody>
</table>

Figure 3: Photograph of callus in brown colour, green bud initiation in between the callus containing 100 ml/l coconut water once they had grown significantly. Following regeneration, before being planted in the field, a full plant generated from somatic embryos was relocated for hardening.

**DISCUSSION**

Plant tissue culture is a technique for removing plant tissues and cultivating them on nutrient media with a number of variables such as propagation, seed type, and media change. The establishment of root culture, regeneration of a whole plant from root culture, and somatic embryogenesis from the cultured roots of the plant *T. cuneifolia* are all demonstrated in this work. First, the application of ruptured and non-ruptured cell was examined initial prior to selection of seeds for embryogenesis. During this process, ruptured cell results in fast germination of seeds and induction of roots and shoot from the seed. Further, with the increase in the level of sucrose in the media, there was an increase in the shoot. 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 (*Bhatia* *et al.*, 2015). *Awad et al.* (2011) explains their study as one of the first which reports regeneration of entire plants using root. Yet again, this is 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 (*Sena*, 2014). *Skoog and Miller* (1957) showed how to obtain a proliferating mass of cells, well understood as *Callus* from plant extracts and subsequently induce the development of roots or shoots by varying the ratio of two important phytohormones (cytokinin and auxin) in the culture medium. 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.

This study also documents one of the most significant findings about the usage of MS semi-solid Medium: profuse shooting was discovered from the roots in culture, which matured into a healthy plant with a success rate of roughly 100% following hardening. On contrary, in liquid media, roots were suspended as cells which formed globular embryolike structures of up to 2mm, brownish to white colour with a smooth rounded appearance. This culture appears as turbid mixture of roots, cells,
cells aggregates and embryos. Now, the embryogenesis and formation of plant from callus depends on several factors, use of media, % sucrose, supplementary and the plants itself are few of those which can be listed through previous studies. Verma et al. (2016) 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 maximum number of somatic embryos in Crocus oliveri. The use of different explants also plays a major role in regeneration and embryogenesis of the plant along with the proportionate addition of auxins (NAA/IAA) and cytokinins (BAP/TDZ) (Vatankhah et al., 2010). Exogenous applications of the hormone initiation during rapid cell proliferation, cell expansion, and differentiation, for example, are known to be influenced by Abscisic acid (ABA). These play a role in cell division and cell cycle control (Francis and Sorrell, 2001).

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 (Awad et al., 2011) explains the possibility of natural Agrobacterium rhizogens infection, which allows the culture to produce its own auxin supplement. 3 percent sucrose grew well and produced somatic embryos in noticeable size, shape, and appearance even in a semi solid medium of 1/4th strength of MS, as shown by Awad et al. (2011) to be specific, cultures in full strength of MS medium tended to induce profuse callus growth while 1/4 strength MS had a higher germination rate, which resulted in a higher yield of somatic embryos and a higher yield of hardened plants. For any of such study, authentication of plant and systematic characterisation are key factors for industrial application of the pilot study experiment. 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 centre would help other researcher’s trial the same and exploit other experiments on the same plant. In this study, root cultures are used for the development of new plant which is considered as useful alternative to clonal propagation and germplasm conservation (Mathur et al., 2014; Rajasekharan and Sahijram, 2015), however, slower growth in the current study could be a downside of the study. The culture also highlights the scope in extraction of glycyrrhizic acid (GA) from Taverniera cuneifolia through HPTLC method and compared with the GA extracts from other plants.

CONCLUSION

From all the above studies, it can be concluded that Glycyrrhiza acid is majorly studied across globe but restricted to extraction by one plant, licorice roots i.e., Glycyrrhiza glabra, however, the present investigation of extraction and evaluation of Taverniera cuneifolia for GA was effectively performed.

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Conflict of Interest:

The authors declare that they have no conflict of interest for this study.

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