Pakistan J. Sci. Ind. Res., Vol. 28 No. 6, December 1985

IN VITRO PROPAGATION OF *RAUWOLFIA SERPENTINA*. THROUGH STEM TISSUE*

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(Received November 26, 1984; September 29, 1985)

Callus cultures were initiated on Murashige and Skoog's medium supplemented with 1% casein hydrolysate, 1 mg/l NAA and 0.5 mg/l kinetin. Buds regeneration was noticed after 20 weeks culture of callus on White's root culture medium containing 100 ml/1 coconut milk, 10 ml/1 biotin, 250 mg/l sodium diethyl-dithiocarbamate and 0.8 mg/l NAA. The plantlets formed roots both with 0.8 mg/l NAA given continuously or with 24 hr. treatment of 3 mg/l each of IAA and IBA. The plantlets established themselves quite easily in soil. For the first few weeks in soil they were nourished with half strength Knop's solution. Afterwards they became completely autotrophic.

INTRODUCTION

Rauwolfia serpentina Benth. ex Kurz. grows wild in India, Bangladesh, Sri Lanka, Burma, Malaysia Thailand and Java in moist deciduous forests ranging from sea level up to 1200 meters. This plant cannot be easily cultivated due to various factors, e.g. formation of non-viable seeds [1], thus limiting its extensive propagation.

Propagation of the plant by means of seeds has so for not been reasonably satisfactory. Other means of propagation are by root stumps and by root, stem and leaf cuttings [2, 3]. Of these the only method which gives a high percentage of successful plants is the propagation by root cuttings [3]. However, the alkaloidal content of roots from plants raised by root cutting is less than those raised from seeds [3]. Furthermore, the roots are not cherished for propagation by the growers due to their demand in drug market.

The cultural techniques are now used an alternative mechanism for the asexual propagation of various plant species [4]. Development of plantlets through induced or regenerated shoot buds has been realized in many plant species including woody plants as the poplar [5], *Picea abies, Pseudotsuga menziesii* [6] and *Citrus sinensis* [7].

Mitra and Chatturvedi [8] reported the differentiation of organized bodies (meristems) in leaf callus explants of *R. serpentina*. Formation of plantlets from stem callus of *R. serpentina* has so far not been reported in literature. Therefore, the present study was undertaken for the purpose of possible rapid multiplication and micropropagation.

MATERIAL AND METHODS

Stem explants of *Rauwolfia serpentina* Benth. ex Kurz. (Apocynaceae) were obtained from plants growing at the Medicinal plants Farm of Pakistan Forest Institute, Peshawar.

For surface sterilization of plant material 1% solution of mercuric chloride was used.

Murashige and Skoog's (MS) medium [9], containing 2% sucrose, was used as culture medium for stem explants. MS contained the vitamins of B-5 medium developed by Gamborg *et al.* [10]. White's Root Culture (RC) medium modified by Street [11] was employed for the culture of the induced stem callus, differentiation of buds and nurturing of buds/plantlets under aseptic conditions. The pH of MS and RC media was adjusted to 5.6.

Regenerated plantlets were watered first with simple tap water and then with the addition of various nutrients in different combinations (Table 1) for first 10 weeks of their growth under incubation. The plantlets, for the next 10 weeks (in incubator) were watered with ½ Knop's supplemented with Berthelot's drops for microelements [12] made in distilled water.

The stock of ENS (Extra Nitrogen Solution) was prepared by dissolving 0.32 g of calcium nitrate and 10 g of potassium nitrate in 100 ml of distilled water.

Agar was used at 0.8% concentration and all the media were sterilized by autoclaving at 15 psi for 15 min. The

* A part of Ph. D thesis submitted to the University of Peshawar by the first author.

Table 1. Effect of various nutrient solutions on growth of stem callus derived plantlets of *Rauwolfia serpentina* in soil for 20 weeks, under 16 hr. light, 28 ±1° temperature and 85 ±5 humidity

Serial No.	Solution constitutes	Observations (numbers indicate week)
1.	Tap water	2nd – General appearance of plantlets almost similar to field grown plants. Leaves mostly green but some slightly yellow at tips. Some growth in the apical buds noticed. Leaves at lower nodes abscissed with sprouting of new leaf buds. 4th – Tips of leaves became yellow, drooped and ultimately fell off.
2.	Tap water + ENS	5th – Leaf abscission not checked.
3.	Inorganic salts (macro and micro) of RC medium dissolved in tap water + ENS	6th – Plantlets regained vigour. Leaves became green but the slight yellowishness of tips still remained. However, further falling off of leaves stopped. New leaves were completely green and the growth in apical buds improved.
4.	Inorganic salts (only macro) of RC medium dissolved in tap water + ENS	7th – Leaves near apical buds seemed protected from further yellowing. 9th – The midribs and veins of leaves turning yellow, otherwise the leaves were healthy, Growth in plantlets, however, continued at slow rate.
5.	½ x Knop's + Berthelot drops (in distilled water).	11th – Leaves acquired complete green colour. The plantlets attained average size of 70 mm. 12th – General appearance of plantlets normal. No discolouration and wilting of leaves. Growth in apical buds continued. Plantlets average size 145 mm.

cultures were kept in controlled 16 hr. cycled fluorescent light, cooled incubators with temperature regulated at 28 ± 1°C, (except otherwise mentioned).

RESULTS

Callus induction: Stem pieces cultured on MS medium with 1000 CH, 1.0 NAA and 0.5 mg/l of K formed callus within 10 days. Callus growth was slow. The callus after 4 weeks was then transferred to MS containing all other factors except that CH was replaced by CM. As a result of CM addition, the callus growth got improved. Therefore, MS was always supplemented with CM in subsequent cultural studies. No bud differentiation occurred on this callus maintained on MS medium even after 25 weeks of culture.

As an alternative some of the actively growing callus was subcultured on RC medium containing 100 ml/l CM, 10 mg/l biotin, 250 SDC and 0.8 mg/l NAA. The callus was subcultured regularly at 4-week intervals on media of the same composition. The callus grew for 20 weeks without shoot differentiation. Moreover this callus, in the first few passages, remained nodular and compact. Thereafter, due to active growth, the new callus on the periphery of the callus inoculums became friable. However, the callus in the inner core, base and at the slow growing portions of the inoculum remained hard and nodular.

Bud differentiation and root formation: After the 5th passage on RC medium (i.e. 22nd week), spontaneous production of numerous meristemoidal structures was noticed in these cultures. Their appearance was like thick and blunt white projections. Only a low percentage of these white meristemoids (cf 20%) grew further and formed well growing green shoots (Fig. 1) within 2 to 4 weeks. Once established in culture, these shoots took a further 4 to 6 weeks period for root formation.

After root formation, the plantlets could easily be separated from the mother callus, cleared of the attached callus and cultured on RC medium containing 100 ml/l CM, 10 mg/l biotin, 1.0 mg/l IAA and a higher dose of a cytokinin, i.e. 10 mg/l BAP (Fig. 2). Four weeks after this treatment, growth in the apical bud was noticed, with the formation of lateral shoots at the basal nodes of these plantlets (Fig. 3). However, after some time the leaves turned yellow and roots developed some callus (Fig. 3). Yellowing of leaves was assumed to be due to less nitrogen in the RC medium as compared to MS (callus induction

medium). Consequently ENS was added at 10 ml/l in all further subcultures. After 5 weeks, the yellowing of the leaves was controlled. The lateral shoots observed earlier from the basal nodes grew profusely and produced branches and more leaves.

Further development of the root system of plantlets was hindered by the formation of the callus. The roots were so much infested with the callus that they were hardly visible. Therefore, the callused roots were excised and plantlets were induced to root anew, by culturing them on RC medium containing reduced amount of cytokinin (2 mg/l BAP), IAA was replaced with 0.8 mg/l NAA; other addenda were the same as on which the plantlets were already growing.

Fig. 1. Shoot differentiation in stem callus cultured on White's Root Culture medium containing 100 ml/l coconut milk (CM), 10 mg/l biotin, 250 mg/l sodium diethyldithiocarbamate (SDC) and 0.8 mg/l NAA.

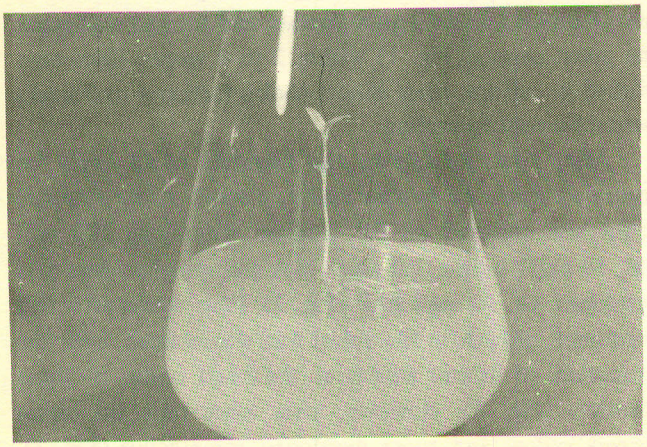

Fig. 2. Roots were formed in 4 weeks on differentiated buds; one such plantlet separated from mother callus is shown in figure.

The plantlets did not produce any root in the subsequent 9 weeks, but their swollen basal portion ruptured and produced green callus. The callus formed at the basal end of the plantlets was separated and the plantlets were then treated with 3.0 mg/l IAA and IBA each for 24 hr. in liquid RC medium. Thereafter, the plantlets on transfer to auxinless liquid RC medium formed roots after one week. The roots were allowed to elongate for 14 days (Fig. 4). These roots did not callus like the previous roots.

Acclimatization. The plantlets after transfer to pots were incubated in 16 h fluorescent illumination. They were also provided with extra illumination (tungsten light by 100 W bulbs per 5 plantlets), for acclimatization to sunlight. The plantlets were watered with simple tap water. They grew quite well for 2 weeks (Fig. 5) and afterwards exhibited deficiency of nutrition by showing signs of yellowing and abscission of leaves. ENS was then given in subsequent waterings at 10 ml/l. Even this treatment did not check leaf abscission. Further modifications of nutrient solution were

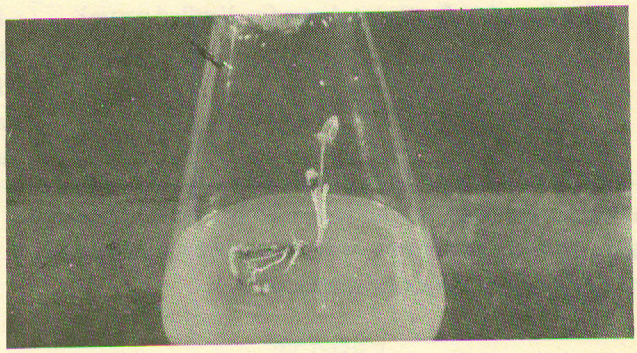

Fig. 3. Formation of lateral shoots and subsequent callus formation on plantlet's root, on White's Root Culture medium containing 100 ml/l CM, 10 mg/l biotin, 1 mg/l NAA and 10 mg/l BAP.

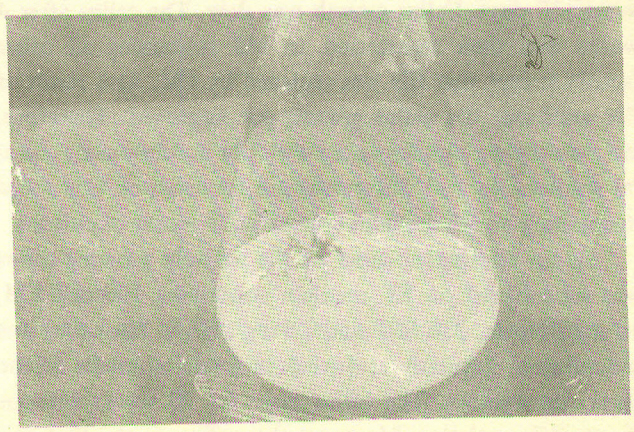

Fig. 4. Root development in 14 days on basal White's Root Culture medium after treatment for 24 hr. with 3 mg/l IAA and 3 mg/l IBA. Roots taken out from the medium and exhibited as exposed to show the full length.

made (Table 1) in 6th and 7th week of plantlet growth. These modifications in nutrient solutions could only assist slow growth in plantlets upto 10th week. Thereafter ½ strength Knop's solution was used. It supported vigorous

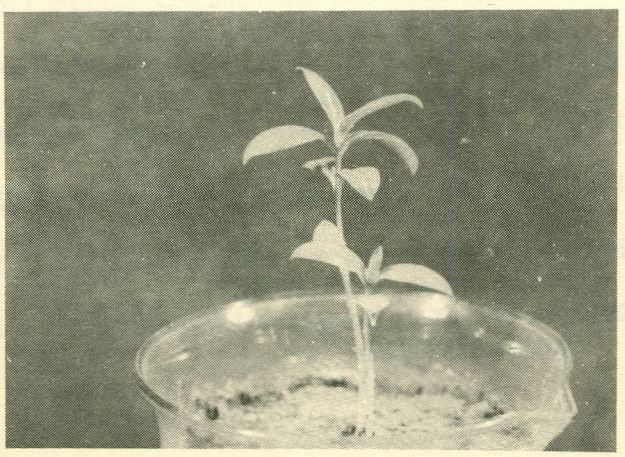

Fig. 5. 2-week-old plantlet growing in soil.

growth in the subsequent weeks. No wilting or discolouration of the leaves was observed during this period. An average increase in plantlets size was noted at 107.14% (Table 1) in 10 weeks. When the plantlets completed 20 weeks in soil, they were taken out of the incubator and kept under varying day and night room temperatures (mid-March) and diffused sunlight through glass window panes. The pots were still covered with polyethylene envelops to retain humidity. The covers were, however, abandoned after 4 weeks in room conditions. After this the plantlets were growing normally in soil. They seemed to have adapted to the changed physical conditions. Moreover, they were fed on simple tap water in ex-incubator period.

DISCUSSION

During the present work, the shoot regeneration in stem callus cultures was obtained on modified White's Root Culture (RC) medium, which was also found suitable for the further development of plantlets. The results varied from crop plants, e.g. sugarcane [13], corn [14], oat [15], and sorghum [16] where MS was preferred for regeneration.

Calli of *Lycopersicon esculentum* [17] which had undergone one or repeated subcultures without shoot formation could not be induced to form shoots. In contrast, during the present investigations, the stem callus of *R. serpentina* regenerated shoot buds only after a subculture period of 20 weeks. It may be the tendency of certain plant tissue to allow differentiation (under otherwise favourable conditions) after a certain period of time. Because 20-25 weeks, after the first subculture, were required before meristematic zones could be observed in *Sinapis alba* [18] and 52 weeks for similar purpose with diploid tissue of *Catharanthus roseus* [19].

The stem callus of *R. serpentina,* which regenerated buds, was heterogenous in texture, being friable at the periphery and top but compact and hard at the base and core. Shoot regeneration in *R. serpentina* from the basal part of inoculated callus piece was similar to the observations made by Ross *et al.* [20], for tobacco callus.

The leaf shedding of field growing plants was complete at the time of transfer of regenerated plantlets to soil in October; it was the main reason that the plantlets were potted and kept at 28° in the incubator. The various nutrient solutions tested were from simple tap water, ENS solution, macro-and micro-salts of RC medium, terminating at half strength Knop's solution supplemented with Berthelot's solution for micro elements. It is, however, recommended that the last nutrient solution (under sequence order in Table 1) could be safely and directly used for watering of plantlets of *R. serpentina* before planting them in field. The preference by plantlets for a simple nutrient solution may partly be due to their ability to manufacture their own food autotrophically. This was confirmed later (after 20 weeks) by the normal growth of plantlets in room conditions on simple tap water.

The plantlets were healthy in look and green like field growing plants. However, the capability of meristemoidal structures, in stem callus of *R. serpentina* to develop into green shoots, was found only 20%. Overcrowding may be one of the reason, because it might be inhibiting other meristemoids to develop into plantlets. Therefore, this technique could only be beneficial if some method is developed to stimulate all or most of such meristemoidal structures to grow into green shoots. The percentage of appearance of such structures was quite satisfactory (220%). Moreover, the rooting capability of green shoots was found 100%. Therefore, the stem callus of *R. serpentina* is suggested as a suitable material for clonal propagation of the species.

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