In vitro propagation of *Rivina humilis* L. through proliferation of axillary shoots and shoot tips of mature plants

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In *vitro* propagation of *Rivina humilis* L. was attempted through proliferation of axillary shoots and shoot tips obtained from mature plants. The shoot tips and nodal shoot segments exhibited 70 and 80% shoot initiation when cultured on Murashige and Skoog (MS) basal medium supplemented with 4.44 μM 6-benzylaminopurine (BAP)+2.85 μM indole-3-acetic acid (IAA) and 8.88 μM BAP+2.68 μM naphthalene acetic acid (NAA), respectively. Shoots (2 to 3) were obtained from both shoot tip and nodal explants on MS medium supplemented with BAP (2.22 μM), IAA (2.85 μM) and silver nitrate (4 μM AgNO₃). Multiplication was maximum on MS medium supplemented with BAP (8.88 μM), IAA (2.85 μM) and AgNO₃ (4 μM) wherein 3-4 shoots per nodal explant were obtained. Microshoot elongation was achieved on MS medium containing BAP (2.22 μM) and gibberellic acid (2.89 μM GA₃). Shoots (3-4 cm) exhibited 100% rooting within 4 wk in medium containing half MS with IAA (2.85-5.71 μM) or IBA (2.45-4.90 μM) alone or in combination with BAP (2.22 μM), resulting in simultaneous rooting and shoot growth. About 70% of micropropagated plants were established successfully in micropots and transferred to the field after 3 months.

Keywords: *In vitro* rooting, *Rivina humilis*, Phytolaccaceae, multiple shoots, nodal explants

Introduction

*Rivina humilis* L. (Family: Phytolaccaceae), popularly known as pigeon berry, grows well in the Caribbean, tropical America and Africa¹, and now widely naturalized in Indo-Malaysia and Pacific islands. It is also found as a neo-tropical plant in the central parts of Taiwan². Seeds are propagated through dispersal by birds. *R. humilis* is widely used as an ornamental plant, while red berries are used for cosmetics. Antibacterial activity of the plant has been reported³. The red berries, rich in betalains (0.3% fresh wt; 1.7% dry wt), were used by native Americans as a red dye. Betalains rich *Rivina* berry juice was also found to be safe in rats⁴. Betalains are accumulated in plants of only 11 families under Order: Caryophyllales⁵. Betalains are functional hydrophilic colourant suitable for food stuffs in pH 4-6 range⁶. In view of the rich betalains content, recently authors have characterized the pigments, nutritional components and antioxidant activity of the berries⁷. In view of these developments, mass multiplication of *R. humilis* is very much required for sustainable production of betalains from its berries.

Plant regeneration through axillary shoot proliferation using nodal explants from mature plants has been reported⁸ and was found to be one of the most promising ways for multiplying a selected variety true to its type, showing the same agronomic characteristics with genetic similarity. Similarly, shoot tip culture has been a powerful technique to eliminate virus infection and thereby resulting in production of healthy and vigorously growing planting material⁹. So far, there is no report on *in vitro* multiplication of *R. humilis* through shoot tip or axillary shoot proliferation. Hence, we report a method for successful and rapid micropropagation of *R. humilis* through shoot tip and axillary shoot proliferation from mature (6-month-old) plants.

Materials and Methods

Ripened fruits of *R. humilis* were collected from 6-month-old plants available on the campus of the institute. The botanical confirmation of the plant was done⁷. Seeds were collected from the fruit pulp, washed 3-4 times with distilled water and then sown in pots containing garden soil after soaking overnight in 2, 3, 4 and 6% urea to increase permeability in seed coat. Within 4-5 d of sowing, the percentage of seed germination obtained was recorded. The germinated seedlings were then transplanted into individual pots for their further growth, and this could reduce the microbial load while preparing the explants, because *R. humilis* plants in general grow under tree shades

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where high litter exists. For explants preparation, 6-month old healthy plant was selected and nodal and shoot tip explants (each ~1 cm in length) were prepared. The explants were washed, treated with Bavistin WP (1%, w/v) for 15 min, mercuric chloride (0.15%, w/v) for 3 min and later rinsed 4-5 times with sterile distilled water.

For shoot induction, the medium containing Murashige and Skoog (MS) basal salts, vitamins, 3% sucrose and supplemented with plant growth regulators (PGRs), such as, 6-benzylaminopurine (BAP: 2.22-8.88 µM), α-naphthalene acetic acid (NAA: 2.68-10.74 µM), kinetin (Kn: 4.65 µM) and indole-3-acetic acid (IAA: 2.85-11.42 µM) either alone or in combination, was used. The medium was adjusted to 5.8±0.2 before gelling with gelrite (0.15%, w/v) for 3 min and later rinsed 4-5 times with sterile distilled water. The explants were washed, treated with silver nitrate (AgNO₃) (0.2%, w/v). All the cultures were incubated at an temperature 25±2°C and 16 h photoperiod (45 µmol m⁻² s⁻¹) using fluorescent tubes (Philips India Ltd, Mumbai, India). Similarly, in another set of experiment, silver nitrate (AgNO₃, 1-15 µM) was incorporated into the MS basal medium containing sucrose (2%), BAP (8.88 µM) and IAA (2.85 µM), and the nodal and shoot tip explants were inoculated onto it. In order to see the influence of different explants, explants having 1-4 nodes were taken. The microshoots obtained on shoot initiation medium were transferred to shoot elongation medium, wherein the combination of BAP (2.22 µM) with indole-3-butyric acid (IBA: 2.45 µM), IAA (2.85 µM) or gibberelic acid (GA₃, 2.89 µM) was tried. Similarly, the microshoots were also transferred to AgNO₃ (1-4 µM) containing medium supplemented with IAA (2.85 µM) and BAP (8.88 µM) at 2% sucrose. The number of shoots, leaves and shoot length was recorded after 8 wk. The newly formed shoots from these primary shoots were further transferred into media containing GA₃ (0.72 µM) and BAP (2.22 µM) for further elongation.

In vitro rooting was initiated by sub-culturing the in vitro shoots of ~4 cm length into ½ strength MS medium supplemented with IAA (2.85-5.71 µM), IBA (2.45-4.90 µM) or NAA (2.65-5.37 µM) alone or in combination with BAP (2.22 µM). Number of roots, root length as well as number and size of the leaves were documented after 8 wk of culture under 16 h photoperiod. The rooted plants were taken out from the culture medium and washed gently with tap water to remove the traces of agar and nutrients. After washing, the plantlets were dipped in 0.05% (w/v) Bavistin and transferred to micropots containing sand, soil and compost (1:1:1) for hardening under greenhouse conditions for 2 months (28±5°C & 80% RH) and then to the field.

To initiate shoots from both shoot tip and nodal segment for each combination of PGRs, 10 explants were used and the experiment was repeated twice. Similarly, for elongation of microshoots and in vitro rooting, 10 explants were used and repeated twice. All the values obtained were expressed as mean±SE. The growth parameters were recorded and the data within each experiment was subjected to one way ANOVA. The comparisons between the mean values of treatments were made by Duncan’s multiple range test.

Results and Discussion

Ex vitro seed germination of R. humilis was found to be the best when the seeds were soaked overnight in 4% urea and then sown in pots; wherein 95% germination was observed in 4-5 d. The seedlings reached a height of 5-6 cm within 15 d. The seeds treated with 2, 3 and 6% urea only recorded 30, 60 and 50% germination, respectively. However, seeds without urea treatment did not germinate till day 5. All the germinated seedlings grew into healthy plants on being transplanted into individual pots. Among these, the best plant (2-month-old) was selected and its nodal and shoot tips were used as explants for further studies.

The morphogenic induction potential of different growth regulators in R. humilis was evident from the results presented in Table 1. The nodal and shoot tip explants showed visible growth and most of them grew into microshoots of 0.8-3.0 cm and 0.6-1.0 cm length, respectively within 30 d. Shoot formation was affected by the concentration of growth regulators used in the medium. Proliferation of shoots was noticed with protuberant appearance from the nodal explants. In 3 wk, microshoots appeared prominently on medium containing BAP along with IAA/NAA at different concentrations (Table 1). Apart from this, granular greenish callus mass was noticed from the base of nodal explants, particularly in combination of BAP (4.44 µM) and NAA (5.37 µM). Similar developmental changes were also noticed in shoot tip explants. Appearance of 1 cm long shoot with 2-4 leaves was observed in 6-8 wk of culture in the medium supplemented with BAP+IAA or BAP+NAA combinations (Table 1). Among different auxin (NAA, IAA) to cytokinin (BAP) ratios used for shoot
initiation from the nodal explants, BAP (4.44 µM) +IAA (2.85 µM) and BAP (4.44 µM)+NAA (5.37 µM) showed good response, in which 80 and 70% nodal explants responded for shoot initiation, respectively (Table 1; Figs 1a & b). A maximum of 2.6 shoots per node with a shoot length of 2 cm and at least 10 leaves were recorded when medium containing BAP (4.44 µM)+IAA (2.85 µM) (Fig. 1a) was employed for shoot initiation. In Kn (2.35-9.30 µM) +IAA (2.85 µM) combination, the response was poor.

Maximum number of shoots per explants from shoot tip (2.6±0.2) and nodal (2.8±0.28) explants were evident after 1 wk in the presence of AgNO₃ (2.63 µM) and IAA (2.85 µM). Similarly, dwarf shoots with long petioles were observed on BAP (2.22 µM)+IBA (2.45 µM) supplemented medium (Fig. 1d). The microshoots attained 2-2.5 cm length in the presence of BAP (2.22 µM) and IBA (2.45 µM) in 6 wk. But, on medium containing BAP (2.22 µM)+NAA (2.65 µM), dwarf shoots with many rosette leaves were produced (Fig. 1e). Upon transferring into shoot elongation medium comprising GA₃ (2.89 µM) and BA (2.22 µM), an increase in the shoot length up to 3-4 cm with 3-4 shoots was observed (Fig. 1f). The shoots, produced from the nodal explants cultured initially on AgNO₃ supplemented medium, were separated and transferred into the same media containing AgNO₃ (4 µM), BAP (8.88 µM) and IAA (2.85 µM) at 2% sucrose. New shoots (2.7±0.27) with 3.41±0.07 cm length were produced on either side of the node with 14.1±0.67 leaves (Fig. 1g).

Among the different auxins tried for in vitro rooting, IAA (5.71 µM) stimulated the best response, wherein 2.6±0.26 roots with a root length of 3.13±0.22 cm were produced. IBA (2.45 µM) also supported in vitro rooting of microshoots, wherein 2.5±0.26 roots with a root length of 1.52±0.22 cm were obtained. Medium devoid of hormones did not support in vitro rooting. The combination of IAA (2.85 µM)+ BAP (2.22 µM) produced 2.2±0.06 roots with 1.58±0.11 cm root length and the number of leaves were found to be 7-10 with a leaf area of ~50 mm² (Fig. 1h). NAA (2.65 µM)+BAP (2.22 µM) was also able to induce rosette like appearance along with in vitro roots of length (~1-1.6 cm) and 30-40 small leaves. Callus was formed in most of the treatments. On addition of hormones like BAP (2.22 µM) and IAA (2.85 µM) into the medium, thick and robust rooting was observed within 10 d of sub-culture. Upon hardening, 70% of plants survived. The plants rooting was observed within 10 d of sub-culture. 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producing shoot buds. In earlier studies, the superiority of BAP over Kn has also been reported\(^1^2\). In the present study, the medium containing AgNO\(_3\) along with IAA and BAP supported direct organogenesis of multiple shoots from nodal explants in \(R. \) humilis. Being an ethylene action inhibitor, AgNO\(_3\) addition to the culture media greatly improved the regeneration of many dicot and monocot cultures\(^1^3\). It was found to be beneficial in regeneration and clonal propagation of many other economically important plants\(^8\) as well as in the somatic embryogenesis of coffee\(^1^4\). Earlier reports showed that silver nitrate promotes shoot formation from nodal explants in \(Vanilla \) planifolia\(^8\), \(Coffea\) sp.\(^1^5\) and \(in vitro\) rooting in \(Decalepis hamiltonii\)\(^1^6\), wherein upto 40 \(\mu M\) AgNO\(_3\) supported shoot formation and \(in vitro\) rooting but, in the present study, even at 4 \(\mu M\) AgNO\(_3\) significant response was noticed. In conclusion, BAP (8.88 \(\mu M\)) and IAA (2.85 \(\mu M\)) along with AgNO\(_3\) (4 \(\mu M\)) favoured multiple shoot induction and the combination of BAP and GA\(_3\) was found to be the best for shoot elongation. This is the first report on micropropagation of \(R. \) humilis.

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**References**


