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In vitro regeneration of shoots and *ex vitro* rooting of an important medicinal plant *Passiflora foetida* L. through nodal segment cultures



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Abstract Methods were developed in the present investigation for cloning and large scale plant production of *Passiflora foetida* L. germplasm selected from the East-Coast region of South India. Nodal shoot segments were used as explants. The explants were dressed and surface sterilized with 0.1% (w/v) HgCl₂. Multiple shoots were induced (6.13 ± 0.22 shoots per explant) by proliferation of nodal shoot meristems on Murashige and Skoog (MS) semi-solid medium + 2.0 mg l⁻¹ 6-benzylaminopurine (BAP). The shoots of *P. foetida* were further multiplied (16.45 ± 0.44 shoots per explant) on MS medium + 0.5 mg l⁻¹ each of BAP and Kinetin (Kin). The *in vitro* generated shoots were rooted on half-strength MS medium containing 2.5 mg l⁻¹ indole-3 butyric acid (IBA). By this method 67% shoots were rooted. About 97% shoots were rooted *ex vitro* (8.33 ± 0.29 roots per shoot) when the cut ends of the shoots were treated with 300 mg l⁻¹ IBA for 5 min. The *in vitro* rooted plants were hardened and acclimatized in the greenhouse and successfully (100%) transplanted to the field.

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1. Introduction

Plant derived natural products such as flavonoids, terpenes and alkaloids have received considerable attention in recent years because of their eco-friendly method of curing ailments [33,24]. *Passiflora foetida* L. (stinking passion flower) which belongs to the family Passifloraceae is an exotic medicinal vine which is extensively used in the Ivorian folk medicine [2]. It is

fast growing and spreading vine, found in riverbeds, dry forest floors, and way side thickets, covering the top of thorny shrubs and also growing near hamlets.

The decoction of *P. foetida* leaves and unripe fruits is used to treat snake bites, women infertility, epilepsy, abscess, hysteria and emmenagogue [21]. The leaf paste is applied on the head for giddiness and headache, icteric, hepatitis, constipation, oesophagy and pains [13]. In Brazil, the herb is used in the form of lotions or poultices for erysipelas and skin diseases [12]. *P. foetida* has been studied to have antispasmodic, sedative, anxiolytic (allaying anxiety), antiparasitic, antibacterial, antifungal and antioxidant activities [4,15]. Furthermore, this

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plant exhibited hepatoprotective, antidepressant, anticarcinogenic, analgesic and anti-inflammatory properties [49,16,27,1].

The important phyto-constituents of *P. foetida* are passifloricins, polyketides, alkaloids, phenols, glycoside flavonoids, cyanogenic compounds and alpha-pyrone [18,14]. About 294 volatile compounds have been isolated so far from the fruit extracts alone of this plant [46].

The use of *P. foetida* in the treatment of women infertility suggests that this plant could have some estrogenic and/or antiestrogenic properties. Since synthetic estrogens are known to cause endometrial or breast cancer and other adverse effects [25,41], the use of plants as new natural sources of estrogens is investigated and encouraged recently [9]. The fiber dimensions and cellulose, hemicellulose study indicated that it could be a suitable material for various grades of paper production [10].

Due to important medicinal properties of this plant it has been exploited by drug manufacturers in India, and the natural population of this plant is decreasing day-by-day due to over harvesting from the open forests [42]. Plant cell and tissue culture techniques could be applied for rapid and mass propagation of medicinal and economic valuable plants. This could be used as an alternate source of large scale cultivation of rare, endemic, threatened and endangered plants [29].

Some reports are available on *in vitro* studies of this plant [38,5,30], but the number of shoots regenerated, *ex vitro* rooting and acclimatization, and the percentage of survival of *in vitro* raised plantlets under field conditions were very limited. We have developed an efficient micropropagation protocol for this valuable medicinal plant which can be used for the conservation strategy of *P. foetida*. The aim of this study is to propagate *P. foetida* in a mass scale to conserve its germplasm through biotechnological interventions.

2. Materials and methods

2.1. Plant material and explant sterilization

The plant material was collected from the East-Coast of South India which includes the coastal Puducherry and Tamil Nadu. The survey was conducted to select the superior and healthy plant material from January to June, 2013. The fresh shoots of the *P. foetida* were collected from different places and brought to the laboratory. Some plants were maintained in the greenhouse to get better and healthy shoots. Nodal shoot segments with one to two nodes (2–3 cm long) were used as explants for the establishment of cultures. Explants were treated with 0.1% (w/v) Bavistin (a systemic fungicide; BASF India Ltd., India) for 5 min. Surface sterilization was carried out with 0.1% (w/v) HgCl_2 (disinfectant, Hi-Media, India) for 5 min under aseptic conditions in laminar air flow cabinet (Technico Systems, Chennai). These were finally rinsed 5–6 times with sterile distilled water.

2.2. Establishment of cultures and induction of shoots

The explants were inoculated vertically on MS medium [32] for culture initiation. Different concentrations of cytokinins (BAP or Kin ranging from 1.0 to 5.0 mg l^{-1}) were incorporated for each in the medium for induction of buds from the explants. These cultures were incubated at $25 \pm 2^\circ\text{C}$ temperature under light (40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ Photosynthetic Photon Flux Density,

PPFD) for 25–30 days. The light was provided by fluorescent tubes in the incubation chamber.

2.3. Multiplication of shoots *in vitro*

The *in vitro* regenerated shoots were multiplied by repeated transfer of mother explants and subculturing of *in vitro* produced shoots on fresh medium. For multiplication of cultures, MS medium supplemented with various concentrations and combinations of cytokinins (BAP and Kin ranging from 0.5 to 3.0 mg l^{-1}) was used. The cultures were incubated at $25 \pm 2^\circ\text{C}$ temperature and 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD for 12 h/d.

2.4. *In vitro* rooting of shoots

For the rooting of *in vitro* produced shoots, the shoots were isolated of appropriate size (2–3 cm) and rooted on full, half and 1/4th strengths of MS basic medium containing auxins (IAA and IBA, ranging from 0.5 to 3.0 mg l^{-1}). The cultures were kept under diffused light (30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD) for one week. After seven days, the cultures were shifted to the normal light conditions for another four weeks. The *in vitro* rooted plantlets were washed with autoclaved distilled water to remove adhered nutrient agar from the roots and then transferred to autoclaved Soilrite® (a combination of perlite with peat moss and exfoliated vermiculite procured from KelPerlite, Bangalore, India) containing bottles for hardening.

2.5. *Ex vitro* rooting of regenerated shoots

Experiments were conducted to test the *ex vitro* root induction from the cut ends of the shoots. The *in vitro* produced long shoots (3–4 cm) were carefully excised from shoot clumps and treated with auxins (IBA and IAA). The bases (4–5 mm) of shoots were treated with different concentrations (50–500 mg l^{-1}) of IBA and IAA for 5 min. Auxin treated shoots were directly transferred to autoclaved soilrite® in bottles moistened with one-fourth strength of MS basal salts. These were directly kept in the greenhouse for rooting as well as hardening of the plantlets.

2.6. Hardening of plantlets in the greenhouse

The *in vitro* rooted plantlets and the auxin treated shoots were hardened by a two step method. In the first stage, the plantlets were kept in the greenhouse for 2–3 weeks in capped glass bottles containing autoclaved soilrite® moistened with 1/4 strength MS salts. The plantlets were gradually exposed to the greenhouse conditions by loosening and removing the caps. In the second stage, the plantlets were transferred to the nursery polybags containing a mixture of sand, soilrite®, organic manure and black soil in a 1:1:1:1 ratio. The successful hardened plantlets of *P. foetida* were transferred to the nursery.

2.7. Observations and data analysis

The cultures were regularly subcultured on fresh medium after 4–5 week interval. The observations were taken after every seven days of inoculation. The experiments were repeated thrice with twenty replicates per treatment. The rate of

multiplication represents the number of shoots produced per explant on a specific medium after the number of days of its inoculation as mentioned in the results. The level of significance with different parameters was determined by variance (ANOVA) using SPSS software (16.0 ver.). The level of variance was analyzed by Duncan's Multiple Range of Test (DMRT) at 0.5% level.

3. Results and discussion

3.1. Establishment of cultures and induction of shoots

The stem of *P. foetida* being hairy required extra care for surface sterilization with HgCl_2 . Maximum number of shoots (6.13 ± 0.22) were induced from the nodal explants on MS medium supplemented with 2.0 mg l^{-1} BAP. The explants harvested during the months of April–June were found most suitable for the initiation of cultures of *P. foetida* (Fig. 1A) and almost all the explants responded. The nodal segments collected during January–March months required longer time (5–6 weeks) to regenerate the shoots and are difficult to sterilize.

The morphogenic response of nodal explants of *P. foetida* was observed in almost both the treatments with cytokinins (BAP and Kin) as shown in Table 1. The explants inoculated on growth regulator free MS medium did not exhibit any regeneration from the nodal meristem even after 40 days of incubation. Nodal shoot segments were used as a tool for *in vitro* propagation of various medicinal plant species such as *P. foetida* [5,38], *Salvadora persica* [36], *Momordica dioica* [45], *Caralluma edulis* [35], etc. Endosperm was also used as explants of *P. foetida* by Mohamed et al. [30].

Cytokinins activate the axillary bud which is present on the nodal region of the explants. Out of the two cytokinins tested in this study, BAP was reported more effective compared to Kin in shoot induction and less number of shoots (4.45 ± 0.72) were proliferated with Kin. The frequency of shoot proliferation from the nodal meristem increased with increasing concentration of the cytokinins up to some extent only. Most of the *Passiflora* species showed good response with BAP during shoot proliferation [17,20,31,47,38,34]. The efficacy of BAP over Kin during shoot initiation has also been reported for

Table 1 Effect of cytokinins (BAP and Kin) on induction of shoots from explants of *P. foetida*.

| Conc. of BAP (mg l^{-1}) | Conc. of Kin (mg l^{-1}) | Number of shoots/explants Mean \pm SD | Response (%) |
|-------------------------------------|-------------------------------------|--|--------------|
| Control (0.0) | (0.0) | 0.00 ± 0.0 | 0 |
| 1.0 | – | 4.56 ± 0.36^c | 82 |
| 2.0 | – | 6.13 ± 0.22^e | 98 |
| 3.0 | – | 5.29 ± 1.03^d | 96 |
| 4.0 | – | 4.35 ± 0.16^c | 91 |
| 5.0 | – | 4.66 ± 0.81^c | 89 |
| – | 1.0 | 2.38 ± 0.87^a | 73 |
| – | 2.0 | 4.45 ± 0.72^c | 86 |
| – | 3.0 | 4.63 ± 0.14^c | 88 |
| – | 4.0 | 3.44 ± 0.67^b | 84 |
| – | 5.0 | 3.16 ± 0.63^b | 71 |

Note: Experiments were conducted three times and twenty replicates were used. Mean separation was analyzed by ANOVA using SPSS software (var. 16.0) and significance variation between the concentrations was studied using DMRT at 0.5% level.

Leptadenia reticulata [40], *Crataeva magna* [8], *Arnebia hispidissima* [44], etc.

3.2. Multiplication of shoots

The shoots were multiplied by subculturing of the proliferated shoots and repeated transfer of mother explants with the shoots. The rate of multiplication of shoots increased up to 5th subculture (Fig. 1B) after that the shoot length was reduced significantly along with vitrification. The increase in number of shoots may be due to suppression of apical dominance during subculture [26,48]. After 4–5th subculture maximum 16.45 ± 0.44 shoots could be obtained from a single node in this study on MS medium augmented with 0.5 mg l^{-1} of each of BAP and Kin (Table 2).

Maximum 2–3 shoots were multiplied using node and shoot tip explants on MS medium supplemented with BAP in the earlier report on *in vitro* culture of *P. foetida* [38]. Isutsa [22] also elongated *Passiflora* shoots with 2.2 mg l^{-1} BAP. Britto et al. [11] used zygotic embryos as inoculums and achieved

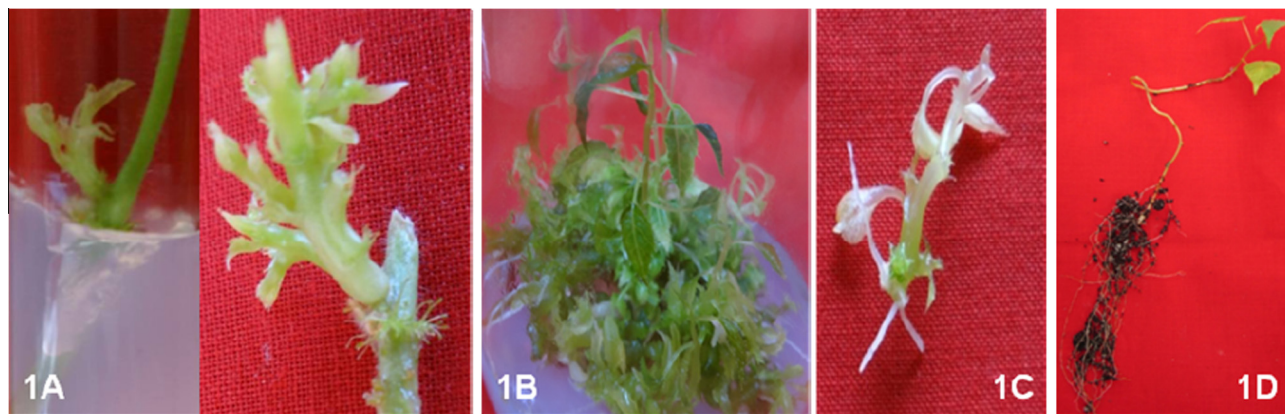


Figure 1 (A) Induction of shoots from the nodal meristem of explants. (B) Multiplication of shoots *in vitro* on MS medium with BAP and Kin. (C) *In vitro* rooted shoot of *P. foetida*. (D) *Ex vitro* rooting of the shoots.

Table 2 Combined effects of cytokinin (BAP and Kin) on multiple shoot formation from sub-cultured shoots on MS medium.

| Conc. of BAP + Kin (mg l ⁻¹) | Number of shoots Mean ± SD | Length of shoots (cm) Mean ± SD |
|--|-------------------------------|------------------------------------|
| Control (0.0) | 0.00 ± 0.0 | 0.00 ± 0.0 |
| 0.5 | 16.45 ± 0.44 ^c | 5.22 ± 0.61 ^c |
| 1.0 | 14.57 ± 0.67 ^d | 5.10 ± 0.43 ^c |
| 1.5 | 11.36 ± 0.23 ^c | 4.51 ± 0.21 ^b |
| 2.0 | 9.56 ± 0.87 ^b | 3.67 ± 0.56 ^a |
| 2.5 | 7.37 ± 0.34 ^a | 3.28 ± 0.21 ^a |
| 3.0 | 7.31 ± 0.65 ^a | 3.12 ± 0.67 ^a |

Note: Experiments were conducted three times and twenty replicates were used. Mean separation was analyzed by ANOVA using SPSS software (var. 16.0) and significance variation between the concentrations was studied using DMRT at 0.5% level.

indirect regeneration of multiple shoots of *P. foetida* in cultures. Anand et al. [5] also induced multiple shoots on BAP and Kin combination. Shoot was also regenerated using mature endosperm culture of *P. foetida* with BAP [30]. Fernando et al. [19] regenerated multiple shoots directly from leaf primordium. Kamathi et al. [23] regenerated multiple shoots of *P. foetida* on MS medium containing BAP and NAA. Our results were similar in terms of the use of BAP and Kin in shoot multiplication, and better in number of shoots regenerated during multiplication stage than the results of earlier workers. Further subculturing (after 6th cycle) resulted in vitrification of shoots. Subculturing was essential within 4–5 week interval otherwise defoliation would have started.

3.3. *In vitro* root induction

Successful rooting in regenerated shoots is the prerequisite to facilitate their establishment in soil. In the present study, the role of different auxins (IBA and IAA) in the root induction was tested. Among the various concentrations of IBA tested, maximum *in vitro* rooting of individual shoots was achieved with half strength semisolid MS medium containing 2.5 mg l⁻¹ IBA (Fig. 1C). Among the different salt strengths of MS salts media tried ½ strength MS medium was found good in terms of percentage of rooting (67%) and mean number of roots (3.00 ± 0.12) (Table 3). Full strength MS medium showed

Table 3 Effect of strength of MS medium augmented with 2.5 mg l⁻¹ IBA on *in vitro* root initiation from shoots of *P. foetida*.

| Strength of MS medium | Response (%) | Number of roots Mean ± SD | Intensity of Callus |
|-----------------------|--------------|------------------------------|---------------------|
| Full strength | 28 | 1.8 ± 0.53 ^a | Moderate callus |
| Half strength | 67 | 3.00 ± 0.32 ^c | No callus |
| 1/4th strength | 51 | 0.67 ^b | No callus |

Note: All the experiments were conducted three times and ten replicates were used. Mean separation was analyzed by ANOVA using SPSS software (var. 16.0) and significance variation between the concentrations was studied using DMRT at 0.5% level.

only 28% rooting after 40 days of inoculation on same combinations of growth regulator. Moderate callus formation was also observed from the basal part of the shoot on full strength MS medium combination.

About 63% shoots were rooted on half strength MS medium augmented with 2.0 mg l⁻¹ IAA (Table 4). Kamathi et al. [23] rooted *in vitro* shoots on the MS medium containing 1.0 mg l⁻¹ IBA and IAA. Anand et al. [5] reported roots directly from the nodal explants of *P. foetida* with IBA and NAA combination. Ragavendran et al. [38] also rooted shoots on MS medium supplemented with 1.0 mg l⁻¹ IBA. Even trace amounts of cytokinin in MS medium cannot enhance any root system from the cut ends of the shoots [28]. Arikat et al. [6] and Shekhawat et al. [43] reported that auxins (especially IBA) play an important role in the induction of roots from the cut ends of the *in vitro* raised shoots of *Salvia fruticosa* and *Turnera ulmi-folia*, respectively.

3.4. *Ex vitro* rooting of the shoots

The percentage of *ex vitro* rooting response was better than the *in vitro* rooting in the present study. The rooting was achieved successfully by treating the shoots with IBA solution and about 97% shoots were rooted when the *in vitro* raised shoots were treated with 300 mg l⁻¹ IBA for 5 min (Fig. 1D). But lower concentration of IBA and all the concentration of IAA resulted in poor development of roots from the micro-shoots. Yan et al. [50] reported that *ex vitro* rooting in micro-propagation technique could overcome the problems during acclimatization prior to transplanting in the field conditions. Maximum 8.33 ± 0.29 roots per shoot were observed during this experiment (Table 5). This is the first report on *ex vitro* root induction in this plant with very good rate of response. *Ex vitro* rooting could enhance the chances of survival of plantlets in the field conditions [7]. It is a cost effective technique and could save labor, time and energy in plant propagation system [50,39].

Table 4 Effect of auxins (IBA and IAA) on *in vitro* induction of roots from the shoots of *P. foetida* on half strength MS medium.

| Conc. of IBA (mg l ⁻¹) | Conc. of IAA (mg l ⁻¹) | Number of roots Mean ± SD | Response (%) |
|------------------------------------|------------------------------------|------------------------------|--------------|
| Control (0.0) | – | 0.00 ± 0.0 | 0 |
| 0.5 | – | 1.02 ± 0.52 ^a | 26 |
| 1.0 | – | 1.34 ± 0.37 ^{abc} | 33 |
| 1.5 | – | 2.53 ± 0.22 ^{ef} | 43 |
| 2.0 | – | 2.86 ± 0.16 ^f | 59 |
| 2.5 | – | 3.00 ± 0.12 ^f | 67 |
| 3.0 | – | 2.72 ± 0.51 ^f | 52 |
| – | 0.5 | 1.24 ± 0.21 ^{ab} | 31 |
| – | 1.0 | 1.66 ± 0.61 ^{bcd} | 39 |
| – | 1.5 | 1.87 ± 0.55 ^{cd} | 47 |
| – | 2.0 | 2.59 ± 0.84 ^{ef} | 63 |
| – | 2.5 | 2.06 ± 0.44 ^{de} | 58 |
| – | 3.0 | 1.62 ± 0.72 ^{abcd} | 42 |

Note: Experiments were conducted three times and twenty replicates were used. Mean separation was analyzed by ANOVA using SPSS software (var. 16.0) and significance variation between the concentrations was studied using DMRT at 0.5% level.



Figure 2 (A–C) Hardening of plantlets in the green house. (D) Hardened plant in the field conditions.

Table 5 Effect of auxins (IBA and IAA) on *ex vitro* root induction from the *in vitro* raised shoots in *P. foetida*.

| Conc. of IBA (mg l ⁻¹) | Conc. of IAA (mg l ⁻¹) | Number of roots Mean ± SD | Response (%) |
|------------------------------------|------------------------------------|--------------------------------------|--------------|
| Control (0.0) | – | 0.00 ± 0.0 | (0.0) |
| 50 | – | 6.02 ± 0.37 ^c | 62 |
| 100 | – | 8.21 ± 1.13 ^f | 76 |
| 200 | – | 7.23 ± 0.71 ^c | 83 |
| 300 | – | 8.33 ± 0.29 ^f | 97 |
| 400 | – | 0.00 ± 0.0 ^a | 91 |
| 500 | – | 6.02 ± 0.37 ^c | 80 |
| – | 50 | 5.84 ± 0.61 ^{bc} | 73 |
| – | 100 | 5.33 ± 0.59 ^b | 76 |
| – | 200 | 6.34 ± 0.21 ^{cd} | 76 |
| – | 300 | 6.88 ± 0.71 ^{dc} | 66 |
| – | 400 | 7.34 ± 0.93 ^c | 83 |
| – | 500 | 6.80 ± 0.44 ^{d^c} | 73 |

Note: Experiments were conducted three times and twenty replicates were used. Mean separation was analyzed by ANOVA using SPSS software (var. 16.0) and significance variation between the concentrations was studied using DMRT at 0.5% level.

3.5. Hardening and acclimatization of plantlets

The hardening of *in vitro* propagated plantlets of *P. foetida* was the crucial step prior to the transplantation of plantlets to the soil. The micropropagated plantlets of *P. foetida* require special treatments for acclimatization. Initially the plants were covered by plastic caps for about 15 days so as to maintain 100% relative humidity. But after 2 weeks the humidity was gradually lowered down by loosening and finally removing the bottle caps. Mean time the plantlets developed an efficient root system, built up new leaves and became photosynthetically active (Fig. 2A).

The *in vitro* plantlets were growing in very good relative humidity conditions and these also depend on the medium for the supply of sugar and other nutrients [3]. Plants are therefore, allowed to grow on rooting media for about 4 weeks after root initiation. During this phase the nutrients in the culture

go on gradually depleting and plantlets become sturdy and easy to acclimatize in the greenhouse [37]. After one month, the micropropagated plants were planted in the potting mixture containing nursery polybags in the greenhouse (Fig. 2B and C). The hardened plantlets were field transferred successfully with 100% survival rate (Fig. 2D). There should be minimum disturbance to the root system when the plantlets were transferred to polybags and the soil conditions.

4. Conclusion

An efficient *in vitro* propagation protocol has been developed for *P. foetida* in the present study. A very good number of shoots were multiplied and almost all the shoots were rooted using *ex vitro* method. It reduced time, energy and cost of production of micropropagated plantlets and increased the chances of survival in the field condition. This protocol can be used for mass scale production of superior and disease free plants which could be replanted in the forest area and supplied to the farmers at reduced cost as standard stock planting material.

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