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Indirect shoot organogenesis of a valuable medicinal plant *Paederia foetida* L. using nodal explants

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ABSTRACT

Paederia foetida L. is an important medicinal herb harboring lots of essential drug producing metabolites and the plant has been going to be endangered due to lack of proper strategies for conservation. Since, indirect organogenesis by tissue culture is considered as the valuable tools for rapid multiplication and improvement of plant genetic resources, application of this technique should be very imperative for the conservation of this valuable and rare medicinal plant. Hence, the research effort was made to develop a suitable protocol for indirect organogenesis *in vitro* using nodal explants of *P. foetida*. After surface sterilization, the explants were submitted to Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of plant growth regulators for showing the performance in terms of callus induction, shoot proliferation and root initiation. Among the surface sterilants used, 0.1% $HgCl_2$ treated for 2 min and 3% NaOCl treated for 10 min showed better performance and maintained 100% and 80% survivability respectively. MS medium supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D) at 1.5 mg L^{-1} showed better performance than others in terms of initiation of callus from nodal explants. In contrast, naphthaleneacetic acid (NAA) at 0.5 mg L^{-1} and 6-benzylaminopurine (BAP) 0.2 mg L^{-1} showed highest rate of callus proliferation with somatic embryos from proliferated callus. During shoot organogenesis, MS medium supplemented with BAP 2 mg L^{-1} showed better results for the regeneration of shoots from embryogenic calli. The shoots derived from callus produced roots by half strength MS medium supplemented with indole-3-acetic acid (IAA). After acclimatization, the plantlets were allowed to ambient condition for further establishment. Our findings claim the establishment of a suitable protocol for indirect organogenesis of *P. foetida* that could be employed for rapid multiplication, conservation and sustainable utilization of *P. foetida* as valuable genetic resource.

Keywords: Callus, conservation, explants, growth regulators, organogenesis, *Paederia foetida*

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1 Introduction

Medicinal plants are the most exclusive source of life saving drugs for the majority of population in the world (Reddy and Reddy, 2008). In Bangladesh, there have been a long historical background for the uses of medicinal plants for therapeutic purposes (Steinmetz, 1961; Blatter et al., 1981; K.M., 2002; Khare, 2008; Haque et al., 2014). *Paederia foetida* L. belonging to the family Rubiaceae, is an important perennial climbing herb usually named as shunk vine or Gandhabadali. The herb is widely distributed throughout the Asia and native to both temperate and tropical Asia, from India to Japan and South East Asia (Reddy and Reddy, 2008). Though plant parts such as leaf, root, bark and fruit are used for medicinal purposes, several reports documented the dual uses of this wild plant as food and medicine (Dutta and Dutta, 2005). The leaf extracts of this plants are successfully used for physical weakness and almost every type of stomach ailment. The extract is also claimed to be useful in curing allergy and jaundice. In rural areas, the juice of the plant is frequently used to remove pain after childbirth (Khanikar, 1995). The plant has enormous pharmaceutical importance harboring secondary metabolites like iridoid glycosides, sitosterol, stigmasterol, alkaloids, carbohydrates, proteins, amino acids and volatile oils (Blatter et al., 1981; K.M., 2002; Khare, 2008).

Though having a lot of potentials, it is a matter of concern that this valuable natural asset is going to be destroyed due to continuous and gradual interference of natural and anthropogenic activities. Along with natural hazards, different anthropogenic activities like over exploitation, rapid urbanization, industrialization, pollution and global warming are very much responsible for destruction of natural habitats in Asian sub-continents which leading to the continuous threats for biodiversity conservation and extinction of potential plant species including valuable medicinal plants. The medicinal plant *P. foetida* have been enlisted as the vulnerable one in many of the states of India (Ved et al., 2008). Likewise, now-a-days many of the important medicinal plants in Bangladesh are going to be destroyed or confronted huge genetic losses for keeping them unattended for a long time. Unfortunately, we don't have complete databases or inventory of rare and endangered medicinal plants by which we can select suitable species to get more attention. Therefore, based on the databases of neighboring country India, and our observation for last couple of years it is assumed that the medicinal plant *P. foetida* is going to be endangered in Bangladesh due of continuous threats of overexploitation and lack of proper strategies to conserve. Hence, considering the importance, suitable conservation strategies should be emphasized to protect and conserve the rare and endangered medicinal plants like *P. foetida*. As the country like Bangladesh is very prone to natural calamities, the *in situ* conservation strategies likely to face lots of hazards which can be resolved by following *in vitro* conservation strategy such as tissue culture.

In recent years, *in vitro* culture techniques have been getting more attention as a potential tools for rapid multiplication and conservation of germplasms of rare, endangered and threatened medicinal plants (Ajithkumar and Seeni, 1998; Prakash et al., 1999). However, the conventional propagation of *P. foetida* could be maintained by both seed and stem cutting but the processes are very slow (Aquilar, 2001) and not quite safe for therapeutic uses. Hence rapid multiplication of *P. foetida* using *in vitro* technique might be essential for conservation and sustainable utilization. Although few efforts have been claimed from direct organogenesis from nodal explants by applying different concentrations and combinations of plant growth regulators (Amin et al., 2003; Srivastava and Srivastava, 2004; Alam et al., 2010), further efforts should be needed for the development of suitable protocol addressing indirect organogenesis from embryogenic callus. Since, indirect organogenesis of shoots from callus phase have tremendous application in rapid multiplication, making somaclonal variation and genetic improvements (Karp, 1995), developing a suitable protocol of indirect organogenesis for *P. foetida* might help conservation, sustainable utilization and genetic improvements of this valuable medicinal plants. Though, a single claim regarding to organogenesis via callus phase in *P. foetida* has been reported in Bangladesh (Hassan et al., 2012) and therefore, more investigations by maintaining efficient sterilization methods and changing hormonal combinations are essentially needed to address the issue.

Considering the above facts, the present study was undertaken to develop a suitable protocol for indirect *in vitro* organogenesis of *P. foetida* using nodal explants with a variety of combinations of growth regulators and to enhance sustainable conservation technique of *P. foetida*.

2 Materials and Methods

2.1 Preparation, sterilization and culture of plant materials

Healthy and juvenile nodal explants (about 1.0 cm in length) were collected from donor *P. foetida* plants grown at the Department of Crop Botany, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU). After collection, the explants were thoroughly washed with running tap water for five to six times, followed by washing with Tween-20 solution for 5 min and distilled water for at least three times . Then the explants were rinsed with 70% ethanol for two to three times and washed with sterile

distilled water for several times. Then the explants were submitted to different concentrations and durations of sodium hypochlorite (NaOCl) or mercuric chloride ($HgCl_2$) and subsequently washed with sterile distilled water and allowed to grow in MS medium (Murashige and Skoog, 1962). After adding 3% sucrose and 0.8% agar to the MS medium, it was adjusted to pH 5.8 and autoclaved at 121 °C for 20 min. For callus initiation, proliferation, and subsequent shooting, MS medium or MS medium supplemented with different concentrations and combinations of plant growth regulators were used. Half strength MS medium either sole or in combinations with growth regulators was used for root initiation. The cultures were incubated for a 16 h photoperiod at 25±2 °C under an illumination of 35-50 μmol $m^{-2}s^{-2}$ provided by white fluorescence lamps (Thirupathi et al., 2013).

2.2 Treatment combinations for indirect organogenesis

Performances of different concentration of NaOCl and $HgCl_2$ were tested to clarify the best sterilants for *P. foetida* nodal explants. MS medium supplemented with different concentrations and combinations of plant growth regulators were used to find out the suitable growth regulator or combination of growth regulators for indirect embryogenesis of *P. foetida* using nodal explants. 2,4 dichlorophenoxyacetic acid (2,4-D), either sole (0.5, 1.0, 1.5 mg L^{-1}) or combined with 6-benzyleaminopurine (BAP) as (0.5+0.2, 1.0+0.5, 1.5+1.0 mg L^{-1}) and napthaleneacetic acid (NAA) combined with BAP (0.5+0.2, 1.0+0.5, 1.5+1.0 mg L^{-1}) were used to basal MS medium for callus induction. Different combinations of 2,4-D and BAP (0.5+0.2, 1.0+0.5, 1.5+1.0 mg L^{-1}) and NAA with BAP (0.5+0.2, 1.0+0.5, 1.5+1.0 mg L^{-1}) were added to the MS medium for showing callus proliferation and embryogenesis. BAP either sole (0.5, 1.0, 2.0 mg L^{-1}) or combined with kinetin (Kn) at various combinations (0.5+0.2, 1.0+0.5, 2.0+1.0 mg L^{-1}) were supplemented to MS medium for showing shoot regeneration from embryogenic callus. indole-3-acetic acid (IAA) was added to the half strength MS medium by maintaining different concentrations (0.2, 0.5 and 1.0 mg L^{-1}) for the initiation of rooting.

2.3 Data collection regarding sterilization and indirect organogenesis

For the experiments of surface sterilization, the cultures were monitored regularly at seven days interval and death of explants either by contamination or by getting toxicity of the sterilants were recorded. At 21 d of culture, % survivability of the explants regarding to the growth regulators was recorded. After maintaining best surface sterilization, the explants were allowed for callusing in MS or MS medium with growth regulators. Before culture, initial weight of explants was recorded. Any swelling started in cultures was treated as callusing and recorded as initiation of callusing. At 30 d of culture, the final weight of the callus was recorded and callus weight gained by 30 d was calculated and compared regarding to the performance of growth regulators. After taking initial weight, the calli were then sub-cultured by maintaining different combinations of growth regulators for further proliferation of callus and inducing embryogenesis. At 30 d of subculture the weight of embryogenic calli were recorded and weight gained by proliferated callus was calculated and compared. After taking initial weight, the proliferated callus with somatic embryos were sub-cultured in MS medium supplemented with different growth regulators for shoot regeneration. At 30 days of subculture, the number of shoots, length and leaf number per shoots were recorded for every treatments. The shoots derived from calli were cultured to ½ MS medium or ½ MS medium supplemented with IAA and initiation of rooting was recorded.

2.4 Data analysis

The experiments were conducted following completely randomized design (CRD). Five replications were maintained for each treatment. The collected data on different parameters were analyzed by Statistix 10 package statistical programme. Mean separation was done using least significant difference (LSD) values with the help of the same software package.

3 Results and Discussion

Shoot organogenesis by tissue culture is very useful for successful and rapid multiplication of mother plants using minimal plant materials. As the technology has been successfully utilized for the conservation of plant genetic resources, the aim of the present effort was made to find out the suitable protocols for rapid clonal propagation, sustainable utilization and genetic improvement of valuable medicinal plant *P. foetida*. We maintained aseptic and controlled environmental condition for culturing of plant materials. We used MS medium supplemented with different concentrations and combinations of plant growth regulators for *in vitro* culture of nodal explants of *P. foetida*. For getting an efficient protocol, standardization of sterilization process using different combination of sterilants and performances of different combinations of growth regulators regarding to callus induction, shoot proliferation from callus and subsequent formation of roots were monitored and respective data were recorded accordingly.

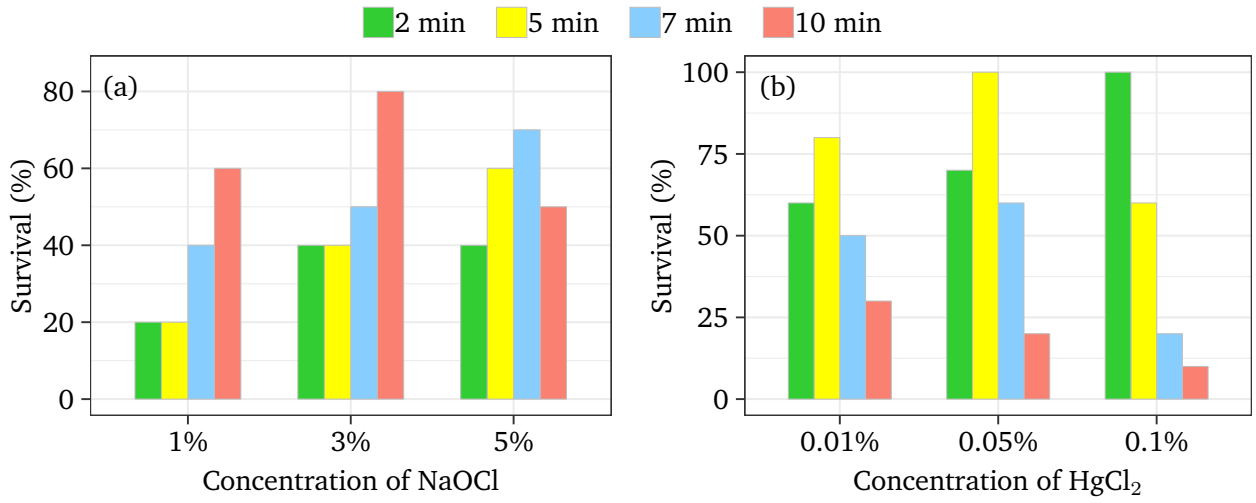

Figure 1. Sterilization of nodal explants by (a) NaOCl and (b) $HgCl_2$ at different concentration and duration of treatments

3.1 Standardization of sterilization of nodal explants of *P. foetida*

As the effective surface sterilants, NaOCl is commonly used in plant tissue culture and compounds of which have the ability to penetrate into the living tissue and kill micro-organisms (Fukuzaki, 2006). $HgCl_2$, another important surface sterilant which is highly antimicrobial and toxic and can kill the micro-organism at very low concentration (Das et al., 2012). Therefore, for the sterilization of nodal explants, we used NaOCl and $HgCl_2$ to grow contamination free plantlets. The effects of different concentrations of NaOCl (1%, 3% and 5%) and duration of treatments (2, 5, 7, and 10 min) are presented in Fig. 1a. The highest percentage of survival (80%) was recorded at 21 d of cultured by maintaining 3% NaOCl treated for 10 min. The rate of survival was further decreased to 50% at 5% NaOCl treated for 10 min indicating increased concentration led to the tissue damage and death (Fig. 1a). The concentration and duration of treatments of surface sterilants may vary depending on the nature of plants either herb or shrub or tree. In case of medicinal plants Aloe vera, 5% NaOCl with 20 min exposure gave above 90% survivability (91.7%) *in vitro* (Sharifkhani et al., 2011). Newman et al. (1996) used 2.5% NaOCl for 15 min for successful sterilization of sugarcane explants. Therefore 3% NaOCl with the exposure of 10 min in our observation likely to be useful for the surface sterilization of nodal explants of *P. foetida*.

Besides NaOCl, utilization of $HgCl_2$ as the surface sterilants is well documented and widely accepted in tissue culture technique (Anburaj et al., 2011; Preethi et al., 2011). Hence, we also used it by maintaining different concentrations (0.01%, 0.05% and 0.10%) and durations of treatment (2.0, 5.0, 7.0, and 10.0 min) and results are presented in Fig. 1b. The highest percentage of survival (100%) at 21 d of culture were recorded when 0.10% and 0.05% $HgCl_2$ treated for 2 and 5 min respectively indicating 0.10% $HgCl_2$ for minimum time duration (2 min) is enough for total decontamination and survival. By further increasing exposure time led to the decreased survival rate due to toxic effect of high concentration of $HgCl_2$ (Fig. 1b). Amin et al. (2003) reported sterilization of shoot tip and nodal explants of *P. foetida* by 0.1% $HgCl_2$ with little bit long exposure time, 10 min, compared to our findings. Whereas, Hassan et al. (2012) used 0.1% $HgCl_2$ for 8 min for surface sterilization of nodal explants of *P. foetida* that is also higher exposure than our findings where 0.1% $HgCl_2$ for 2 min or 0.05% $HgCl_2$ for 5 min maintained above 80% survivability. Since $HgCl_2$ has the higher level of toxicity to plant tissue, care should be needed for optimization of concentration and exposure time during sterilization of explants (Kataky and Handique, 2010). Therefore, low concentration with little exposure time should be very economic and less injurious for living tissue. Although combined application of sterilants has been reported by Thirupathi et al. (2013) where they utilized 0.1% $HgCl_2$, 5% NaOCl and 0.1% cetrimide for sterilization of *P. foetida* explants, the sole application of either NaOCl or $HgCl_2$ is enough to maintain surface sterilization of nodal explants effectively.

3.2 Initiation of callus from nodal explants

Induction of callus or undifferentiated mass of meristematic tissue in plant is regulated by maintaining different plant growth regulators *in vitro* (Valizadeh et al., 2007). Although auxin plays major role, the combination of different growth regulators are also

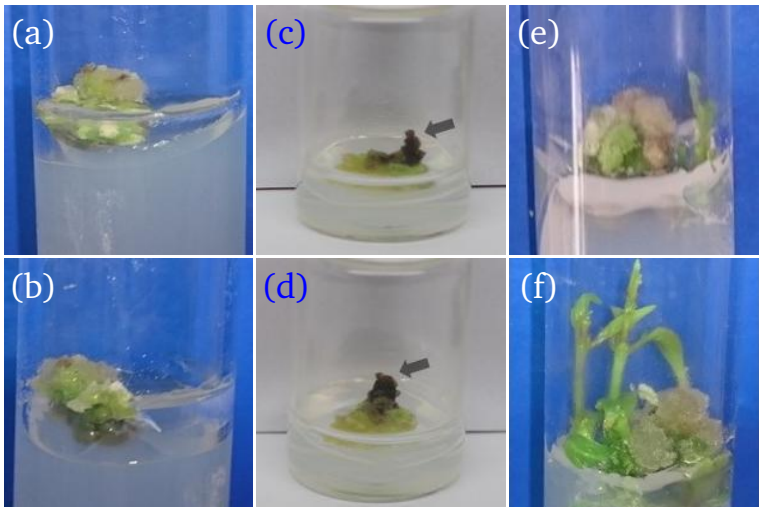

Figure 2. Indirect shoot organogenesis in *P. foetida* from nodal explants. Induction of callus in MS medium supplemented with 2,4-D at (a) 1.5 mg L^{-1} and (b) 1.0 mg L^{-1} at 30 d of culture. Proliferation of callus with somatic embryos enhanced by MS medium supplemented with NAA + BAP at (c) 0.5 and 0.2 mg L^{-1} and (d) 1.0 and 0.5 mg L^{-1} respectively at 30 d of sub-culture. Arrows indicate the development of somatic embryos. Initiation of shoots from embryogenic callus at (e) 15 d and (f) 30 d of sub-culture induced by 2.0 mg L^{-1} BAP.

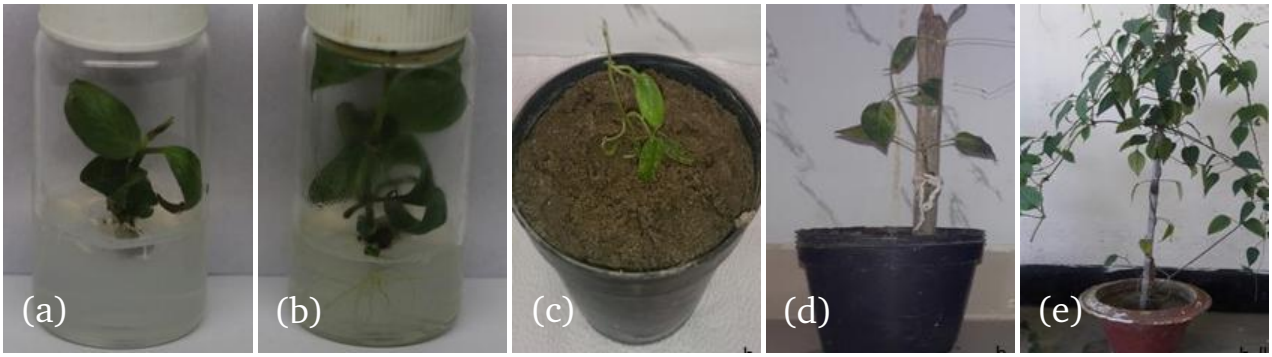

Figure 3. Rooting and acclimatization of plantlets developed from indirect shoot organogenesis. No rooting was observed when plantlets were cultured in sole ½ MS medium at 21 d of culture (a), but medium supplemented with IAA at 0.5 mg L^{-1} produced rooting (b). After acclimatization, the plants were allowed to grow in ambient condition and found to be established. The photos (c, d, e) were taken at 15, 30 and 90 d of planting, respectively.

effective in callus initiation (Martin, 2004). In our efforts, nodal explants of *P. foetida* were cultured in MS medium supplemented with different concentrations and combinations of growth regulators either alone or in combination such as 2,4-D (0.5, 1.0 and 1.5 mg L^{-1}); 2,4-D + BAP (0.5+0.2, 1.0+0.5 and 1.5+1.0 mg L^{-1}); NAA + BAP (0.5+0.2, 1.0+0.5 and 1.5+1.0 mg L^{-1}). Significant differences of weights of callus initiated from nodal explants in responses to plant growth regulators were recorded at 30 d of nodal culture.

The gain of callus weight was found to be highest (0.12±0.007) g when culture was maintained by the growth regulator 2,4-D in basal MS medium at the rate of 1.5 mg L^{-1} followed by (0.11±0.02 g) at 1.0 mg L^{-1} 2,4-D (Fig. 2a,b, Table 1). This indicates better performance of 2,4-D for the callus induction in *P. foetida* and finding of which was supported by Teshome and Feyissa (2015). Though combined application of 2,4-D + BAP (0.5+0.2 mg L^{-1}) showed relatively better performance, the other combinations such as 2,4-D + BAP (1.0+0.5 and 1.5+1.0) mg L^{-1} and NAA + BAP (0.5+0.2, 1.0+1.5 and 1.5+1.0 mg L^{-1}) did not show effective results for callus initiation from nodal explants (Table 1). Hassan et al. (2012) reported the initiation of callus effectively from nodal explants of *P. foetida* when MS medium was supplemented with 1.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA within three weeks of culture . In our observation 2,4-D plays better role than that of BAP and NAA in callus induction from nodal explants indicating suitability of the application of 2,4-D in the callusing of nodal explants of *P. foetida*. Though there was no earlier report of sole application of 2,4-D mediated callus induction in *P. foetida*, the tremendous effect of this growth regulators was reported in other plant species such as highest percentage of callus induction was reported by 2.0 mg L^{-1} 2,4-D in *Ipomoea batatas* (Bett et al., 2019). Similar trend of result regarding to the increased callusing by 2,4-D in *Brucea mollis* was claimed by Das et al. (2017). The present findings reported higher callusing by 1.5 mg L^{-1} 2,4-D which are consistent to those findings. Hence, our results suggest the suitability of the sole application of 2,4-D (1.0 or 1.5 mg L^{-1}) or in combination with BAP (0.5+0.2 mg L^{-1}) for effective callus initiation from nodal explants of *P. foetida*.

Table 1. Effect of plant growth regulators on the induction of callus from nodal explants

Treatments †	Initial weight (g) ‡	Weight at 30 days (g)	Difference of weight (g)
Control	0.0258±0.008 ab	0.0359±0.007 e	0.0102±0.0009 d
2, 4-D (0.5)	0.0197±0.005 ab	0.103±0.03 bc	0.0833±0.03 bc
2, 4-D (1.0)	0.0228±0.004 ab	0.1334±0.02 ab	0.1105±0.02 ab
2, 4-D (1.5)	0.0356±0.01 a	0.1624±0.01 a	0.1268±0.007 a
2, 4-D + BAP (0.5 + 0.2)	0.0242±0.006 ab	0.1067±0.009 bc	0.0825±0.006 bc
2, 4-D + BAP (1.0 + 0.5)	0.0162±0.001 ab	0.0526±0.009 de	0.0363±0.007 d
2, 4-D + BAP (1.5 + 1.0)	0.0223±0.002 ab	0.0446±0.008 e	0.0223±0.007 d
NAA + BAP (0.5 + 0.2)	0.0249±0.007 ab	0.1019±0.006 bc	0.0770±0.008 bc
NAA + BAP (1.0 + 0.5)	0.0156±0.004 b	0.0902±0.009 cd	0.0746±0.004 c
NAA + BAP (1.5 + 1.0)	0.0218±0.004 ab	0.0503±0.004 de	0.0285±0.0006 d

† The value in parenthesis with the name of a plant growth regulator is its concentration (mg L^{-1});
‡ Values of plant growth parameters are mean±SE (standard error).

Table 2. Effects of growth regulator combinations on the proliferation of callus with somatic embryos

Treatments †	Initial callus weight (g) ‡	Callus weight at 30 days (g)	Callus weight gain (g)
Control	0.0141±0.001 ab	0.316±0.04 d	0.30 ±0.04 d
2, 4-D + BAP (0.5 + 0.2)	0.0145±0.003 a	0.885±0.07 ab	0.87±0.07 ab
2, 4-D + BAP (1.0 + 0.5)	0.009±0.0007 bc	0.59±0.04 c	0.58±0.04 c
2, 4-D + BAP (1.5 + 1.0)	0.013±0.002 ab	0.584±0.02 cd	0.57±0.03 cd
NAA + BAP (0.5 + 0.2)	0.010±0.0006 abc	1.08±0.09 a	1.08±0.09 a
NAA + BAP (1.0 + 0.5)	0.007±0.0006 c	1.04±0.05 a	1.04±0.2 a
NAA + BAP (1.5 + 1.0)	0.011±0.001abc	0.688 ±0.02 bc	0.68±0.02 bc

† The value in parenthesis with the name of a plant growth regulator is its concentration (mg L^{-1});
‡ Values of plant growth parameters are mean±SE (standard error).

Table 3. Effect of plant growth regulators on the regeneration of shoots from embryogenic callus

Treatments †	No. of shoot ‡	Shoot length (cm)	No. of leaf
Control	0.00±0.0 d	0.00±0.0 d	0.00±0.0 c
BAP (0.5)	1.00±0.58 cd	0.67±0.35 cd	1.67±0.88 b
BAP (1.0)	1.67±0.33 abc	1.00±0.15 cd	1.33±0.33 bc
BAP (2.0)	2.67±0.33 a	3.00±0.58 a	4.00±0.58 a
BAP + Kinetin (0.5 + 0.2)	1.33±0.33 bc	1.33±0.33 bc	2.33±0.33 b
BAP + Kinetin (1.0 + 0.5)	1.33±0.33 bc	2.67±0.67 a	2.33±0.33 b
BAP + Kinetin (2.0 + 1.0)	2.33±0.33 ab	2.33±0.33 ab	2.67±0.33 ab

† The value in parenthesis with the name of a plant growth regulator is its concentration (mg L^{-1});
‡ Values of plant growth parameters are mean±SE (standard error).

3.3 Callus proliferation with somatic embryos

After initiation, callus proliferation with somatic embryos are the prerequisite for further regeneration of shoots. Therefore, performance of nodal calli were observed under subsequent sub-culturing in MS media supplemented with the combined application of auxin and cytokinin which have great roles in producing embryogenic callus (Robinson et al., 2009). We found proliferated calli with gray color embryos at 30 d of sub-culture showing indication for the regeneration of further shoots (Fig. 2c,d). We did not count the embryos but took the final weights of proliferated callus. We calculated the data of callus weight gained by 30 d of subculture. Significant differences in the weights of embryogenic callus were observed in responses to growth regulators at 30 d of sub-culture (Table 2). The highest mean value of proliferated callus weight (1.08±0.09 g) was found in the MS media supplemented with NAA + BAP (0.5+0.2 mg L^{-1}) followed by (1.04±0.05 g) with NAA + BAP (1.0+0.5 mg L^{-1}) and there was no significant difference in those combinations (Table 2). Findings also suggest the formation of embryogenic callus by the application of auxin and cytokinin in another medicinal plant *Emilia zeylanicaon* using MS medium supplemented with Kn (0.50 mg L^{-1}) and 2, 4-D (0.10 mg L^{-1}) (Robinson et al., 2009) and the results of which is more or less consistent to our results. Whereas, MS medium supplemented with 2,4-D (1.0 mg L^{-1}) was most effective for the induction of somatic embryos in another important medicinal plant *Holostemma ada-kodien* (Martin, 2003). With those findings and our results suggest the varied roles of growth regulators in the formation of embryogenic callus which depends on genotypes, age and nature of explants, protocols and respective culture conditions.

3.4 Shoot regeneration from embryogenic calli

To check the indirect shoot regeneration from proliferated callus of nodal explants, the embryogenic calli were sub-cultured in MS medium by maintaining different combinations of growth regulators specially by maintaining the combination of BAP and Kn which have great roles for the organogenesis from callus (Alam et al., 2010). In our study, sub-culture was followed by MS medium supplemented with BAP (0.5, 1.0, 2.0 mg L^{-1}) and BAP + Kn (0.5+0.2, 1.0+0.5, 2.0+1.0 mg L^{-1}). Total number of shoots, shoot length and total number of leaves at 30 d of sub-culture were recorded and found significant statistical differences in all the parameters mentioned. The highest mean value of shoot number (2.67±0.33) was found when BAP was used alone with the basal medium at 2.0 mg L^{-1} (Fig. 2e,f, Table 3) followed by (2.33±0.33) in the combination of BAP and Kn (2.0+1.0) mg L^{-1} (Table 3). Likewise, shoot length in the same culture resulted the highest (3.0±0.58) value by the use of BAP (2.0 mg L^{-1}). The combination of BAP and Kn (1.0+0.5 and 2.0+1.0 mg L^{-1}) also gave statistically similar output with values (2.67±0.67) and (2.33±0.33) respectively (Table 3). The highest mean value of total leaf number (4.0±0.58) at 30 d of culture was observed at BAP (2.0 mg L^{-1}) followed by (2.67±0.33) at combination of BAP + Kn (2.0+1.0 mg L^{-1}). Considering all the characteristic of shoot, the sole application of BAP (2.0 mg L^{-1}) showed better performance as compared to other combinations. Regeneration of high frequency of shoots from the calli of nodal explants was also reported in *P. foetida* (Hassan et al., 2012) at 6 wk of culture by 0.5 mg L^{-1} BAP and concentration of which is lower than that the concentration used in the present effort although they followed prolonged culture strategy. This is likely to be happened by the variation of physiological maturity of explants and differences in the culture period. High morphogenetic efficiency of calli derived from nodal segments was also reported by Martin (2002). However, sole application of BAP should be useful for successful shoot proliferation from embryogenic calli of *P. foetida*.

3.5 Initiation of rooting in *P. foetida* induced by IAA

The plantlets developed by indirect organogenesis were transferred to the rooting medium maintaining ½ strength MS with auxin supplements. It was reported that initiation of roots was induced in the plantlets of *P. foetida* in ½ strength MS medium supplemented with either IAA or IBA or NAA at (Amin et al., 2003). Hence, for showing rooting performance, we used only single rooting inducer (IAA) in half strength MS medium at different concentrations and found effective initiation of rooting by 0.5 mg L^{-1} IAA, whereas no rooting was observed in ½ MS medium without hormone supplementation (Fig. 3a,b). Other concentrations of IAA also produced roots (data not shown) indicating prominent roles of auxin in the rooting of shoots regenerated by indirect embryogenesis in *P. foetida in vitro*. The earlier report indicates the acceleration of rooting in *P. foetida* by auxin IBA (Amin et al., 2003; Alam et al., 2010). Along with these, our results suggest both IAA and IBA should be useful for root initiation in *P. foetida in vitro*. The rooted plantlets were gently separated from the agar medium and individually transferred to plastic pots filled with sand, soil and vermi-compost for acclimatization. Then the acclimatized plants were transferred to the large pots and allowed to further growth at ambient condition which were found to be successfully established (Fig. 3d-e).

4 Conclusions

Based on the findings of the present efforts in indirect organogenesis of *P. foetida*, it can be concluded that sterilization of nodal explants by 3% NaOCl for 10 min or 0.1% $HgCl_2$ for 2 min should be useful for maintaining 80% or more survival rate. MS medium supplemented with 2,4-D at 1.5 mg L^{-1} should be very useful for callus initiation from nodal explants. Whereas, combination of NAA at 0.5 mg L^{-1} and BAP 0.2 mg L^{-1} was found to be most effective for callus proliferation with somatic embryos. MS medium supplemented with BAP 2 mg L^{-1} showed better performance for the regeneration of shoots from embryogenic calli. However, half strength MS medium supplemented with IAA suggests the roles of auxin in the rooting of shoots developed from indirect organogenesis of *P. foetida*. Although the protocol which we claimed here likely to be useful for large scale multiplication, sustainable utilization and genetic improvement of medicinal plant *P. foetida*, further efforts should be needed using other plants part such as buds, internodes, leaves, roots etc. and comparison of which with the performance of nodal explants are needed to fine tune the process.

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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