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Original Paper

Multiple shoot bud induction and plant regeneration studies of *Pongamia pinnata*

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Abstract *Pongamia pinnata* is a legume plant which has great potential to be used as a biofuel feedstock. Conventional propagation of *P. pinnata* was found to be inefficient for mass propagation. Employing plant tissue culture techniques for micropropagation and further plant improvement of *P. pinnata* will be the right path to fulfill future challenges in biofuel production. This study aimed to establish a plant regeneration system for potential micropropagation and genetic manipulation of *P. pinnata* in future. In vitro nodal explants were used and Woody Plant Medium (WPM) containing 30 μM 6-benzylaminopurine (BAP) and 1 mM phloroglucinol (PG) was able to induce higher frequency of multiple shoot buds compared to other media investigated in this study. For shoot regeneration study, WPM containing 15 μM of zeatin and 1 mM PG was able to induce longer shoots while rooting of the regenerated shoots was enhanced by WPM supplemented with indole-3-butyric acid (IBA) in combination with silver thiosulphate (STS). A simple and effective acclimatisation protocol was established with very high survival frequency of regenerated plantlets. Root nodulation of the successfully acclimatised plants was also observed. In short, multiple shoot buds were successfully induced, regenerated and rooted in vitro. The rooted plantlets were successfully acclimatised and grown healthily. It was concluded that a successful plant regeneration protocol of *P. pinnata* was achieved for potential application in micropropagation and genetic manipulation.

Key words: biofuel, multiple shoot buds, phloroglucinol, plant regeneration, *Pongamia pinnata*, root nodulation.

Introduction

Fossil fuels, such as oil and natural gas, are the main energy source in the world. However, with limited sources and the high demand of fossil fuels, especially in transportation industry, depletion of fossil fuels is expected in near future (Shafiee and Topal 2008). The depletion of fossil fuels has induced intensive research and efforts to seek for alternative energy sources (Dorian et al. 2006). Studies on bioenergy which can be used for heat, electricity and fuel has shown very good potential as a renewable energy source (Yuan et al. 2008). Bioenergy is a renewable energy that can be obtained or derived from biological sources, such as plants, algae or microorganisms. For example, biodiesel can be produced through transesterification reaction using vegetable oils or animal fats in the presence of a catalyst (Marchetti 2011). As edible oils, such as palm oil or soybeans, are usually used as raw materials for biofuel production, large scale biofuel production may cause global imbalance of food supply due to the competition between the demands from biodiesel and food productions. To avoid this, a non-edible oil crop is the preferred alternative source for biofuel production (Ahman et al. 2011).

Various types of non-edible oil crops are chosen as the candidate for biodiesel production, for examples, *Jatropha curcas*, *Pongamia pinnata*, and *Calophyllum inophyllum*. These plants are known to have high oil production, hence, they are suitable to be used as the biofuel feedstock. *P. pinnata* was chosen for this study because its seeds produce 25–40% oil, which is comparable to that of high oil content crops, such as oil palm and *J. curcas* (Divakara et al. 2011). Besides, *P. pinnata* is a fast growing leguminous tree which has the ability to undergo nitrogen fixation through symbiosis (Azam et al. 2005; Belide et al. 2010; Biswas and Gresshoff 2014; Scott et al. 2008). This symbiotic relationship enables *P. pinnata* to grow with reduced application of fertilisers, at the same time resulting in reduced planting cost and nitrogen pollution (Biswas and Gresshoff 2014; Divakara et al. 2011). In addition, it is suitable to be cultivated widely as it is able to withstand a wide range of stresses such as water logging, slight frost, high salinity conditions, and unfertilised soils types, such as stony sandy and clayey soils (Ahmad et al. 2009).

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Conventionally, *P. pinnata* is propagated through seed germination and grafting or cuttings. However, these conventional methods are time consuming, and face limitations, such as low germination rate, shallow rooting and shortage of high quality planting materials (Azam et al. 2005; Sugla et al. 2007). Therefore, these methods are not efficient for mass propagation of plants. In addition, constraints such as long juvenile period (4 to 7 years), and infection by pests also affect propagation of *P. pinnata* (Orwa et al. 2009). In order to promote *P. pinnata* to be the premium feedstock for biofuel industry, it is important to study the propagation protocol and genetics of the plants to optimise the yield of oil production (Scott et al. 2008). For instances, establishment of micropropagation system and molecular marker-based characterisation to aid the process of elite plant selection of *P. pinnata* have been carried out by a number of researchers. Various explants such as de-embryonated cotyledons (Sujatha et al. 2008), cotyledonary nodes (Belide et al. 2010; Shrivastava and Kant 2010; Sugla et al. 2007) and axillary meristems or nodal segments (Kesari et al. 2012; Sujatha and Hazra 2007) were investigated for their potential in plant regeneration. Embryogenesis was also achieved using callus culture (Mahmood and Zia-Ul-Hasan 2013). However, the frequency of regeneration can be further improved by studying different combinations of phytohormones. Thus, further improvement on the plant regeneration system of *P. pinnata* is required. With the establishment of an effective tissue culture protocol, genetic engineering of the plant can also be performed to improve traits, such as disease resistance, yield and quality of oil besides micropropagating the selected elite plant.

Materials and methods

Plant materials

Seeds of *Pongamia pinnata* were collected from trees growing in Kampar, Perak in this study. The collected seeds were sterilised and germinated in vitro. The nodal explants of the germinated plantlets were used for subsequent studies.

Medium and culture condition

Woody Plant Medium (WPM) (Lloyd and McCown 1980) was used as the basal medium in this study. Macro and micro salts and vitamins were added accordingly with 3% (w/v) sucrose and 0.28% (w/v) Gelrite. The pH value of the medium was adjusted to 5.7 prior to autoclaving at 121°C and 15 psi for 15 min. All plant growth regulators were filtered sterilised and added accordingly after autoclaving in this study. All cultures were kept at 25±1°C with 16 h photoperiod (1000 lux) and 8 h darkness.

Multiple shoot bud induction from nodal explants

A preliminary screening of different types of cytokinins were carried out. Nodal explants at the size of 5 mm were excised from in vitro plantlets and cultured on WPM supplemented with 5 μM of cytokinin, thidiazuron (TDZ), BAP, zeatin or N^6-(2-Isopentyl) adenine (2IP). WPM without supplemented with PGR was used as control. The experiment was repeated twice with four replicates, five explants per replicate, for each repeat.

Based on the results obtained, TDZ and BAP were selected to optimise shoot bud induction at various concentrations (10, 20, 30, 40, 50, and 100 μM). WPM without supplemented with PGR was used as control. Subsequently, to further enhance multiple shoot bud induction, WPM incorporated with 30 μM BAP (based on result obtained from the above experiment) in combination with 1 mM phloroglucinol (PG) or 1 mM glutamine was investigated. WPM supplemented with 30 μM BAP was used as control. The experiments described above were repeated twice with triplicate using five explants in each replicate.

Regeneration of induced shoot bud

Zeatin and 2IP were selected to study shoot bud regeneration based on the results obtained from the multiple shoot bud induction experiment. Shoot buds were excised from the induced one-month multiple shoot buds and cultured on WPM supplemented with zeatin or 2IP at various concentrations (5, 10, 15, and 20 μM). WPM without PGR was used as control. Subsequently, to further optimise, WPM supplemented with 15 μM zeatin in combination with or without 1 mM PG were used to study the effect of PG in enhancing shoot regeneration. These experiments were repeated twice with triplicate for each repeat. Each replicate was cultured with five explants.

Root induction

A preliminary study on root induction was performed using WPM supplemented with different concentrations (5, 10, 20 and 30 μM) of IBA. The results obtained showed 20 μM IBA was the most suitable concentration. Therefore, IBA at 20 μM was selected to investigate the effects of sodium thiosulphate (STS) solution as the additive for root induction from the regenerated shoots. WPM without supplemented with phytohormone, WPM supplemented with 20 μM IBA, and WPM supplemented with and 20 μM IBA in combination with 200 μM STS were investigated. This experiment was repeated twice with ten regenerated shoots for each repeat (a total of 30 regenerated shoots).

Acclimatisation

Acclimatisation of *P. pinnata* was carried out using the regenerated plantlets. The plantlets were taken out from the culture bottles and washed thoroughly with tap water to remove residues of the medium. Individual plantlet was transferred into a culture bottle containing vermiculite moistened with tap water. The transferred plantlets were kept at room temperature (27°C) for one month. Then, the covers

of the cultures bottle were removed, and the plantlets were kept at room temperature (27°C) for another month. Finally, these plantlets were transferred and grown in pots under shade. The growth medium used was a mixture of soil, vermiculite, and perlite with 1 : 1 : 1 ratio. The experiment was repeated twice, 10 rooted plantlets for each repeat. A total of 30 in vitro plantlets were used in this studied. Observation was performed and the survival frequency of acclimatised plantlets was recorded.

Histological analyses

Histological analyses were performed on the induced shoot buds. The sample was prepared according to the reported protocol with minor modifications (Liu et al. 2009). The sample was fixed for 24 h in the formalin–acetic acid–alcohol (in ratio of 1 : 1 : 18) solution before dehydrating in a gradual series of tertiary-butyl alcohol (TBA) solutions. The sample was then immersed in molten of paraffin wax for 24 h before embedding in paraffin wax on a metal mold. The solidified wax embedded with the sample was then sectioned into 10 μm thickness using a microtome (Leica RM2235). The sectioned samples were then transferred to a water bath at 55°C and then mounted onto slides. The waxed sample on the slide was dewaxed in absolute xylene solution for 3 min thrice followed by rehydrating in a gradual series of ethanol solutions, 3 min in each solution. The hydrated tissues were stained with 0.5% (w/v) haematoxylin for 3 min and then rinsed with distilled-water thrice prior to stain with 0.5% (w/v) eosin for 30 s. The stained samples were then observed under light microscope.

Statistical analysis

Data recorded were analysed using one-way ANOVA in SPSS 17.0. Significant differences between groups were compared according to Tukey's HSD test at a significance level of 0.05.

Results

Multiple shoot bud induction from nodal explants

In this study, the shoot induction frequency from in vitro nodal explants cultured in the media supplemented with different types of cytokinin (zeatin, 2IP, TDZ, or BAP) was scored (Figure 1a). Very low shoot formation (15.0±10%) was observed from the explants cultured in the control (basal WPM). With incorporation of cytokinin, increment on the frequency of shoot induction was observed. The medium supplemented with TDZ induced highest frequency of shoot formation (96.7±1.2%) followed by the medium added with BAP (66.73±15.3%), zeatin (60.0±21.8%) and 2IP (43.3±7.6%). Although TDZ was able to induce highest frequency of shoot, all the mean length of the shoot formed was only 0.1±0.0 cm after 4 weeks. In contrast, the media supplemented with 2IP, zeatin and BAP were able to induce significantly longer shoots, 0.7±0.2 and 0.9±0.2 and 0.3±0.1 cm, respectively, including the control medium (0.8±0.3 cm).

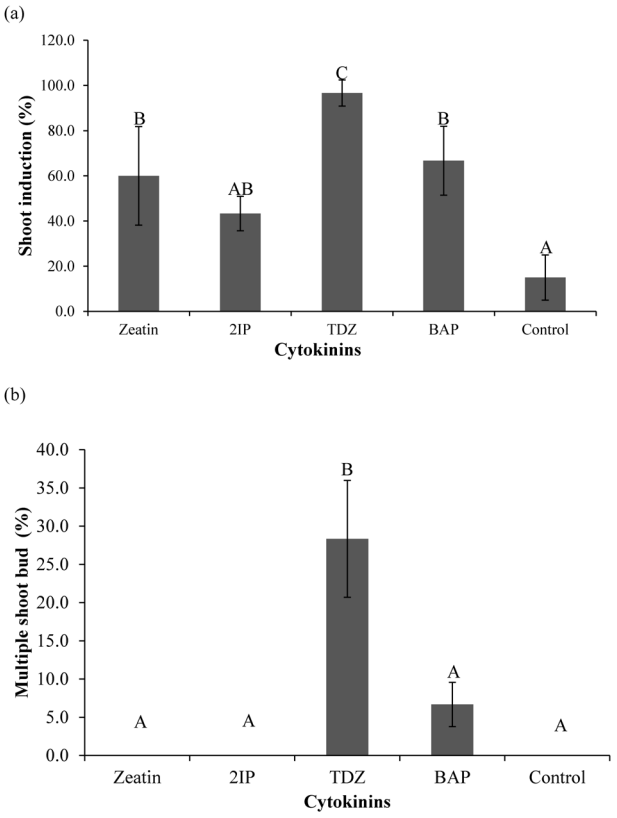

Figure 1. Effects of different types of cytokinins at 5 μM on shoot induction after 4 weeks. (a) Shoot induction (b) Multiple shoot bud induction. Treatment with same letters are not significantly different (Tukey's HSD test at $p=0.05$). Bars indicate standard deviation.

Besides, multiple shoot bud formation was also observed in this study (Figure 1b). The results showed that only TDZ and BAP were able to induce multiple shoot buds. In contrast, the control medium and the media supplemented with zeatin and 2IP were only able to induce single shoot. Highest multiple shoot bud induction (28.3±7.6%) with a mean of 1.3±0.1 shoot buds per explant was achieved using WPM supplemented with 5 μM TDZ. On the other hand, WPM supplemented with 5 μM BAP was able to induce only 6.7±2.9% multiple shoot bud with a mean of 1.1±0.0 shoot buds per explant. In short, the media supplemented with TDZ and BAP were able to induce multiple shoot buds while incorporation of zeatin and 2IP into the media was able to induce longer shoot from the nodal explants. Thus, BAP and TDZ were used for the subsequent multiple shoot bud induction study while zeatin and 2IP were used for subsequent shoot bud elongation and regeneration study.

To optimise multiple shoot bud induction, various concentrations of BAP and TDZ (0, 10, 20, 30, 40, and 50 μM) were investigated. In this study, BAP induced same frequency of multiple shoot buds at 10 and 20 μM (22.2%). The highest frequency of multiple shoot bud induction was achieved at 30 μM (51.1±19.3%).

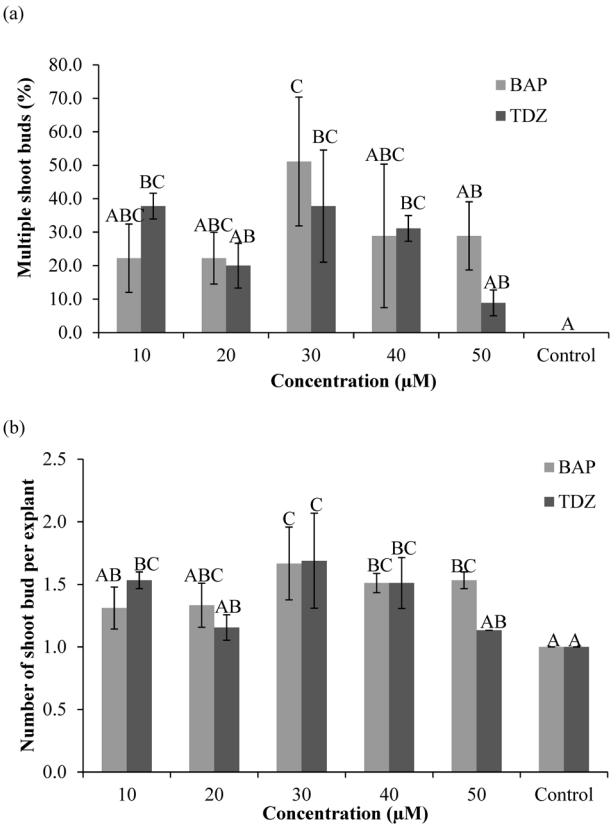

Figure 2. Effects of different concentrations of BAP and TDZ on multiple shoot bud induction after 4 weeks. (a) Multiple shoot bud. (b) the mean number of shoot bud per explant. Treatment with same letters are not significantly different (Tukey's HSD test at $p=0.05$). Bars indicate standard deviation.

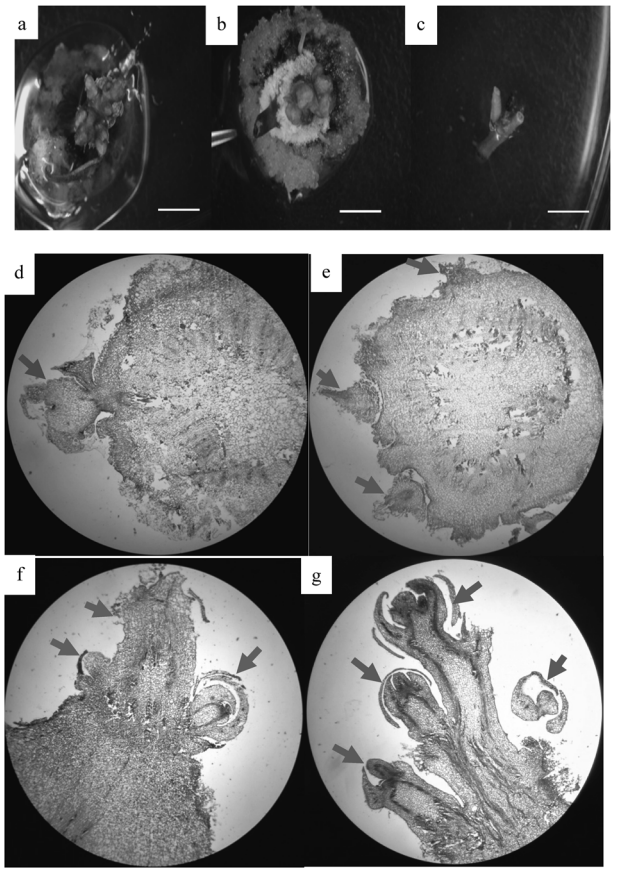

Figure 3. Multiple shoot buds induced by WPM supplemented with BAP and TDZ after 4 weeks and histological observation of cross section nodal explants. (a) 30 μM BAP. (b) 30 μM TDZ. (c) Control (basal WPM). (d) A meristem shoot bud. (e) Multiple shoot bud formed after 1 week. (f–g) Elongation of shoot buds. Arrows indicate induced shoot buds. Scale bar=5 mm. The slides are observed using Leica microscope using 100× magnifications.

Further increment in concentration to 50 μM inhibited multiple shoot bud induction, less than 10% (Figure 2a). As for TDZ, the highest frequency of multiple shoot bud induction was 30 μM and similarly, the highest concentration used (50 μM TDZ) inhibited multiple shoot bud induction, 8.9±3.9%.

Higher number of shoot buds per explant was induced from 30 μM for both BAP and TDZ when they were compared with other concentrations investigated (Figure 2b). The medium supplemented with BAP achieved 1.31±0.2 and 1.33±0.2 shoot buds per explant at 10 μM and 20 μM, respectively. The highest mean number of shoot buds per explant (1.67±0.3) was scored when 30 μM BAP was supplemented into the medium. Further increment in concentration to 40 μM and 50 μM BAP resulted in lower mean number of shoot bud per explant, 1.51±0.1 and 1.53±0.1, respectively. Soft yellowish calli were observed from basal of the nodal explants cultured in the media incorporated with BAP (Figure 3a). As for TDZ, multiple shoot buds were induced regardless of concentration used. The highest mean number of shoot bud induced per explants (1.69±0.4) was observed at 30 μM TDZ. Further increment of concentration to 50 μM led to a lower number of shoot bud induced (1.13±0.0). Similar to BAP, calli were induced in the media containing TDZ (Figure 3b).

To further enhance the efficiency of multiple shoot bud induction, effects of PG and glutamine were investigated. Figure 4a shows that by supplementing 1 mM PG along with 30 μM BAP, the frequency of multiple shoot bud induced was significantly enhanced (48.9±3.8%). The mean number of shoot bud per explant induced was also the highest (1.8±0.1). From the results, WPM supplemented with 1 mM PG was able to further enhance the efficiency of multiple shoot bud induction. The results showed that the medium incorporated with 1 mM glutamine induced very low (4.4±3.8%) shoot bud frequency comparing to control (WPM supplemented with 30 μM BAP), 33.3±0.0%. Similarly, a lower mean number of shoot bud per explant (0.9±0.2) was obtained comparing to the control medium (1.3±0.1). Histological study of different stages of multiple shoot bud induced from WPM in combination with 30 μM BAP and 1 mM PG were performed (Figure 3d–g). Multiple shoot buds were clearly shown as indicating by the presence of

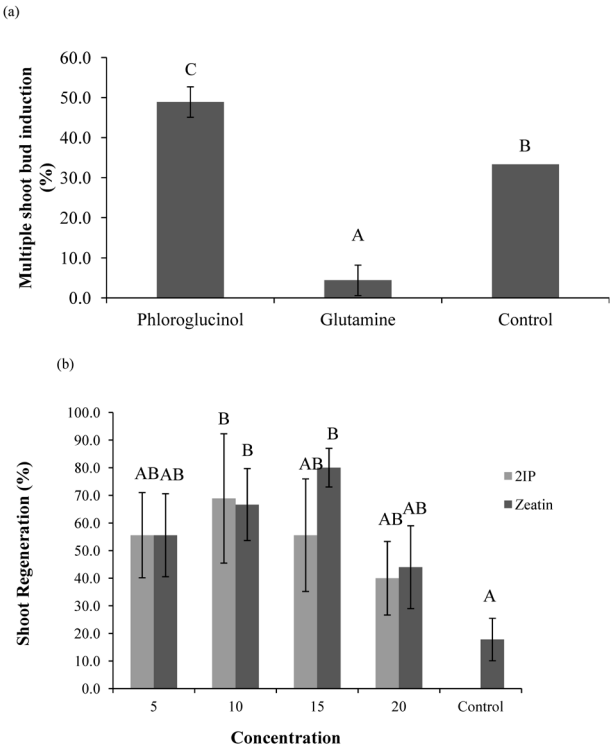

Figure 4. (a) Frequency of shoot bud induction using WPM supplemented with 30 μM BAP in combination with 1 mM phloroglucinol or 1 mM glutamine after 4 weeks. (b) Different concentrations of zeatin and 2IP were investigated for shoot regeneration from the induced buds after 4 weeks. Treatment with same letters are not significantly different (Tukey's HSD test at $p=0.05$). Bars indicate standard deviation.

meristematic regions with actively dividing cells. The elongated shoot was also observed from the four-week culture.

Shoot regeneration

Different concentrations of zeatin and 2IP (5, 10, 15 and 20 μM) were used to investigate shoot regeneration from the induced buds. Figure 4b shows that the highest frequency of shoot regeneration (80.0±7.0%) was scored using 15 μM of zeatin among all the media investigated. While for 2IP, the highest frequency of shoot regeneration was 68.9±23.4%. There were no significant differences in mean length of shoot regenerated among the concentrations of 2IP and zeatin tested. The longest mean shoot length was obtained from the medium supplemented with 15 μM zeatin, 1.0±0.2 cm. By increasing the concentration of zeatin to 20 μM, it reduced the mean shoot length to 0.6±0.2 cm. For 2IP, 0.7 to 0.9 cm of shoot length was obtained from the medium supplemented with 2IP. Although there were no significant difference among different concentrations of zeatin and 2IP, results showed that 15 μM zeatin induced the highest frequency of shoot bud regeneration with the longest shoot length within four weeks. To further enhance frequency of shoot regeneration, the

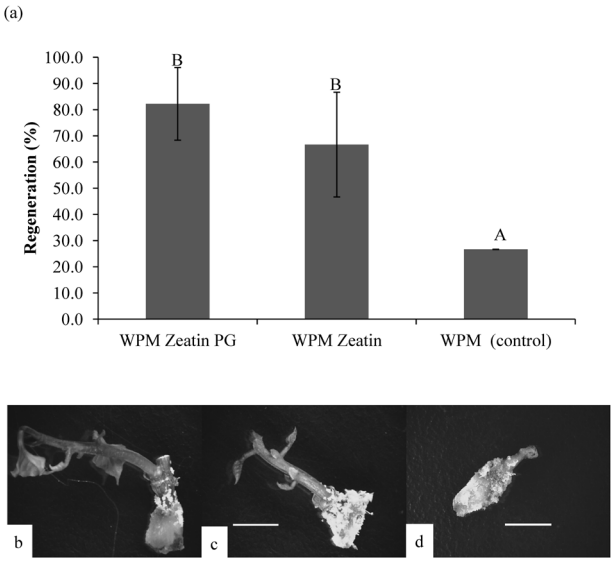

Figure 5. Regeneration frequency of induced multiple shoot buds cultured in WPM, WPM containing zeatin and WPM supplemented with zeatin in combination with phloroglucinol after 4 weeks. (a) Shoot bud regeneration frequency. Treatment with same letters are not significantly different (Tukey's HSD test at $p=0.05$). Bars indicate standard deviation. (b) 15 μM zeatin and 1 mM phloroglucinol. (c) 15 μM zeatin. (d) WPM only. Scale bar=5 mm.

medium supplemented with 15 μM zeatin and 1 mM PG was investigated. The results obtained (Figure 5a) showed that the medium supplemented with 1 mM PG and 15 μM zeatin was able to induce 82.2±14.0% of shoot bud regeneration. In contrast, the medium containing 15 μM of zeatin only obtained 66.7±20.0% of shoot bud regeneration. Besides, the longest mean shoot length was obtained by WPM containing PG and zeatin, 1.2±0.3 cm comparing with the shoots regenerated using WPM, the control medium (Figure 5b–d). However, statistical analysis did not show significant difference in shoot regeneration with the addition of PG.

Rooting of the regenerated shoot buds and acclimatisation

The effects of 20 μM IBA and 200 μM silver thiosulphate (STS) on rooting of the regenerated shoots was carried out. From the results obtained (Figure 6a), WPM in combination with 20 μM IBA and 200 μM STS induced highest frequency of rooted explants, 46.7±5.8%. Other than inducing higher frequency of rooting, WPM in combination of 20 μM IBA and 200 μM STS was able to induce more branched roots. Therefore, WPM supplemented with 200 μM of STS and 20 μM IBA was selected as the root induction medium.

Acclimatisation was performed to the well rooted shoots regenerated from the induced shoot buds before transplanting to the field. A well rooted shoot was kept in covered culture bottles and watered with tap water during the adaption process for four weeks. At the first week, some leaves turned yellow and fell. Three weeks

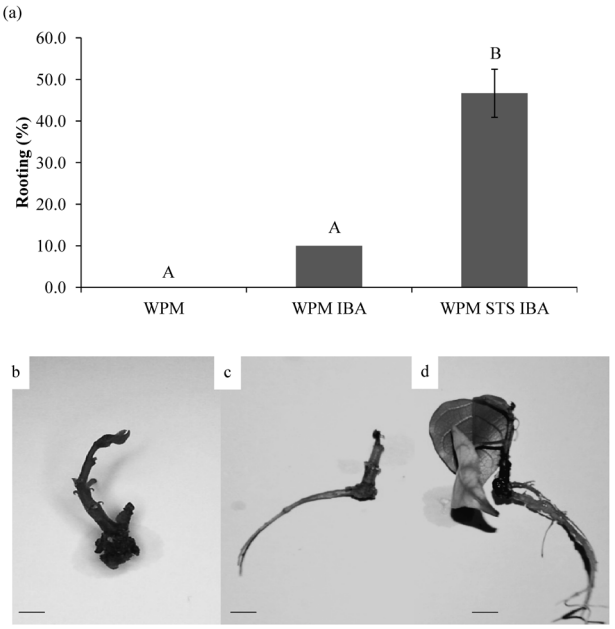

Figure 6. Root induction on regenerated shoots using different media after 4 weeks. (a) Frequency of root induction using different type of media. Treatment with same letters are not significantly different (Tukey's HSD test at $p=0.05$). Bars indicate standard deviation. (b) Basal WPM. (c) WPM supplemented with 20 μM IBA. (d) WPM supplemented with STS and 20 μM IBA. Scale bar=5 mm.

after transferring the plantlets, formation of new leaves were observed and all plantlets survived after 4 weeks. Subsequently, the covers of the culture bottles were removed and the plants were watered weekly with tap water for another four weeks. Similarly, all plantlets survived and they were transferred to pots containing planting medium consisting of vermiculite, perlite and dark soil at 1 : 1 : 1 ratio. All the transferred plants (100.0%) were able to survive and grew rapidly after one month (Figure 7a–c). New leaves were observed and the stem of the plants turned brownish two weeks after transferring to the planting medium. The plants achieved a mean height of approximately 23.0±6.1 cm after one month in the planting medium.

All plantlets that were obtained from in vitro did not show any morphological differences when they were compared to the seed germinated plants growing in the field. The roots of in vitro regenerated plants were compared with seed germinated plants (Figure 7d). It was observed that all seed germinated plants had lesser microroots compared to the in vitro regenerated plants. All in vitro regenerated plants had denser roots compared to the root system of seed germinated plants. Besides, nodules were observed from all the in vitro regenerated plants, but they were not observed in the seed germinated plants.

Discussion

Multiple shoot bud induction from nodal explants

Plant regeneration study was carried out using in vitro nodal explants and cultured on the media supplemented with single cytokinin. The results showed that BAP and TDZ were able to induce multiple shoot buds while zeatin and 2IP were more efficient in shoot regeneration from nodal-stems. In contrast, the control without supplemented with PGR was unable to induce multiple shoot buds and ineffective for shoot regeneration. The results showed that the incorporation of cytokinin into the medium significantly affecting morphogenesis of nodal explants. Each nodal bud has one meristematic dome in the axil, thus, it has higher potential for plant regeneration with the application of cytokinin (Sujatha and Hazra 2007). Cytokinin is commonly used to facilitate multiple shoot bud induction or shoot regeneration. The results of this study showed that BAP and TDZ induced highest multiple shoot buds at the concentration of 30 μM. However, BAP was able to induce higher multiple shoot bud frequency compared to TDZ. In previous studies, multiple shoot buds or multiple shoots of *P. pinnata* were induced from nodal segments (Kesari et al. 2012) and cotyledonary nodes (Nagar et al. 2015a, 2015b) using BAP. Besides BAP, TDZ was also able to induce multiple shoot buds or multiple shoots from different explants such as deembryonated cotyledon (Sujatha et al. 2008), cotyledonary node (Belide et al. 2010), axillary meristem (Sujatha and Hastra 2006; Sujatha and Hazra 2007), embryo and hypocotyl explants (Humánez et al. 2011; Sharma et al. 2011). BAP concentrations ranging from 7.5–22.19 μM were reported to be suitable for shoot multiplication of *P. pinnata* obtained from cotyledonary nodes (Shrivastava and Kant 2010; Sugla et al. 2007) and nodal explants (Kesari et al. 2012; Sujatha and Hastra 2006). However, TDZ was observed to suppress the sprouting and differentiation of *P. pinnata* buds (Sujatha and Hazra 2007; Sujatha et al. 2008) as well as other plant species, such as tamarind (Metha et al. 2004) and banana (Makara et al. 2010). We are unable to compare the efficiency of multiple shoot bud induction with other research groups, as they reported their results in the form of percentage of shoot development (87%; Sugla et al. 2007), shoot regeneration (64–90%; Belide et al. 2010) and explants responded (97.78%; Kesari et al. 2012) together with the mean number of bud per explant. Furthermore, the variations of the results obtained between our study and these studies could also be due to the differences in genotype, type and physiological state of explants, phytohormone and growth medium that were used in the study (Kesari et al. 2012). Besides, the sample size used among these studies were also different.

Subsequently, 30 μM BAP was used in combination

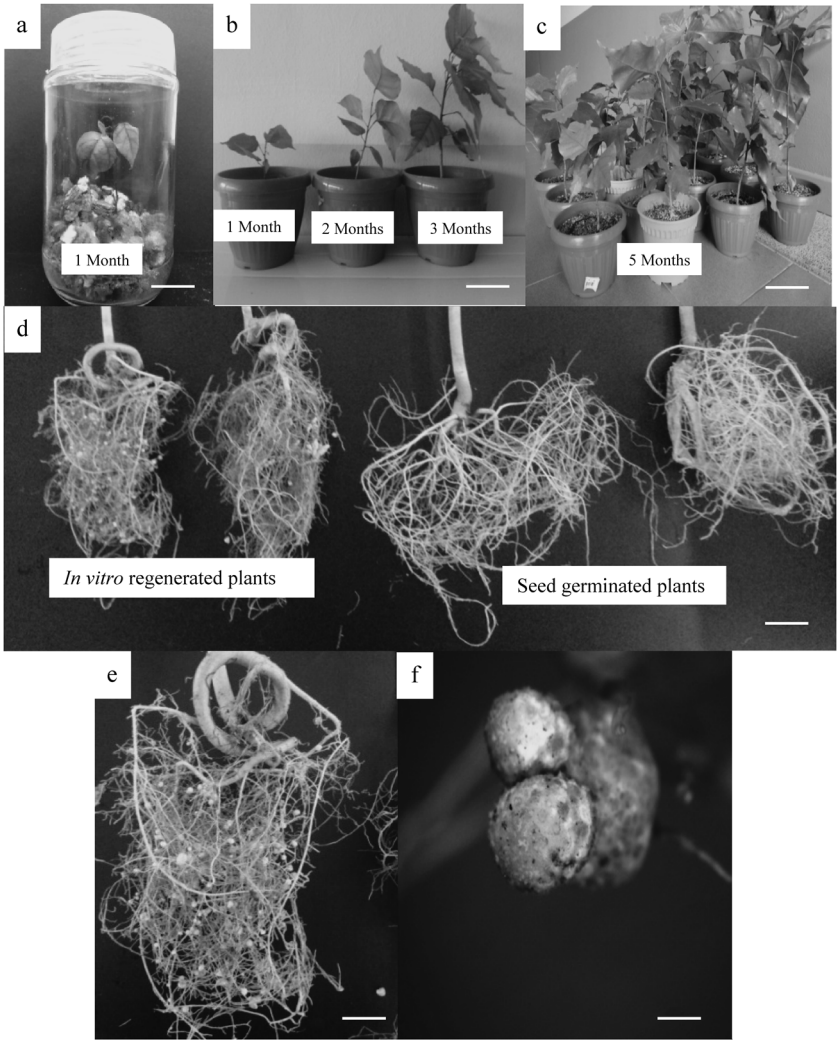

Figure 7. Acclimatization of *P. pinnata* and healthy plants grown in pots. (a) A rooted plantlet in a covered culture bottle; Scale bar=2 cm. (b) Different size and age of plantlets; Scale bar=10 cm. (c) Well established plants; Scale bar=12 cm. (d–e) Nodules are observed on the roots of in vitro regenerated *P. pinnata*; Scale bar=6 cm. (f) Nodules on the roots; Scale bar=0.2 cm.

with either glutamine or phloroglucinol (PG) in the induction medium to further enhance multiple shoot bud induction. Glutamine is usually added into a culture medium as a source of nitrogen (Selvaraj et al. 2007). In this study, the incorporation of glutamine did not enhance *P. pinnata* shoot bud induction frequency. Similarly, incorporation of glutamine did not induce significant improvement in multiple shoot bud induction for *P. pinnata* cotyledonary nodes (Belide et al. 2010). At present, no study on the effects of PG on *P. pinnata* multiple shoot bud induction is reported. PG is a phenolic compound which is able to promote somatic embryogenesis, shoot proliferation, shoot formation and rooting in many plant species (Silva et al. 2013; Siwach and Gill 2011; Steephen et al. 2010). It could be applied singly or in combination with cytokinin for shoot development and proliferation at the concentrations varied from 39.65 μM–1.6 mM in various plant species (Gururaj et al. 2004; Silva et al. 2013). In this study, PG was also able to enhance the shoot bud induction and number of shoot bud induced.

Shoot regeneration

To enhance shoot regeneration, investigation on different concentrations of zeatin and 2IP was performed. Both 2IP and zeatin were reported for their effectiveness in shoot regeneration. For example, 2IP was reported for its strong shoot regeneration effects on multiple shoot induced from leaf explants of *Scrophularia takesimensis* Nakai, a type of woody plant (Jeong and Sivanesan 2015), while zeatin showed higher shoot growth rate on *Vaccinium corymbosum* compared to 2IP (Clapa et al. 2012). Plant regeneration of *P. pinnata* was performed using different approaches in previous studies. For examples, the induced buds were regenerated using the medium supplemented with 8.8 μM BAP (Shrivastava and Kant 2010; Shrivastava and Kant 2011), PGR-free medium (8–12 passages) (Sujatha et al. 2008)

and the application of auxin–cytokinin combination (BAP+NAA) (Kesari et al. 2012). In addition, zeatin was reported to be ineffective in enhancing sprouting efficiency from axillary meristem explants of *P. pinnata* (Sujatha and Hazra 2007). In this study, 15 μM zeatin showed higher frequency in shoot regeneration of induced shoot buds and mean shoot length. Similarly, zeatin was applied on various plants, such as strawberry (Haddadi et al. 2013) after shoot multiplication for shoot regeneration and elongation. The differences in observation might due to different basal media and culture conditions applied.

Besides, additives such as adenine sulphate, PG and glutamine are reported as one of the factors which influences the efficiency of shoot regeneration (Siwach and Gill 2011). The application of PG along with zeatin in this study showed shoot bud regeneration in a shorter period with no laborious subculturing process if compared to the protocol reported by Sujatha et al. (2008). PG was reported for its enhancing effects on shoot formation in several horticultural and grain crops (Silva et al. 2013). Addition of PG into medium improved shoots bud regeneration and longer mean shoot length was observed in this study. Similar efficient additive activity was reported for other plant species, such as *Hedychium coronarium*, longer shoots and higher shoot number were induced by incorporating PG into the medium (Verma and Bansal 2013). PG has both cytokinin and auxin like-activities. The synergetic effects of PG with the phytohormone was observed for shoot, somatic embryo and root development for several fruit, woody and ornamental species (Jani et al. 2015; Silva et al. 2013). It can be applied singly or in combination with cytokinin as both stimulate shoot induction and development, or in combination with auxin for subsequent root induction.

Rooting of the regenerated shoot buds and acclimatisation

Auxin is known for its function to regulate root growth and development (Goh et al. 2014). IBA is an effective auxin for adventitious root formation and was also applied for rooting of *P. pinnata* (Kesari et al. 2012; Shrivastava and Kant 2011). Investigations of the effects of auxins on rooting of *P. pinnata* stem cuttings and in vitro regenerated shoots showed that IBA was more effective compared to other PGRs (Belide et al. 2010; Kesari et al. 2008, 2012; Shrivastava and Kant 2010; Sugla et al. 2007). However, high concentration of auxin will lead to inhibition of rooting, such as rooting from cuttings (Kesari et al. 2008) and regenerated shoots of *P. pinnata* (Kesari et al. 2012).

To further enhance rooting, the effect of silver thiosulphate (STS) was also evaluated on the regenerated shoots. The incorporation of silver in the form of silver nitrate or STS is able to increase the frequency of root induction (Petrova et al. 2011) and improve rooting of microcuttings (Klerk et al. 1999). The incorporation of silver into the medium improve rooting by reducing the oxidative stress through inhibition of ethylene activity and the sensitivity of ethylene (Karni et al. 2010). Incorporation of silver was reported to enhance rooting in various plant species, such as *Gentiana lutea*, *Corymbia maculate* and *Rotula aquatica* lour (Chithra et al. 2004; Petrova et al. 2011; Steinitz et al. 2010) but inappropriate concentration of silver might suppress root elongation and cause browned or blacken roots (Steinitz and Bilavendran 2011). The results showed that the incorporation of 200 μM STS and 20 μM IBA into the medium was able to enhance the rooting in this study.

Acclimatisation was then performed by transferring the in vitro well rooted shoots to the culture vessels containing moistened vermiculite for eight weeks before transplanting to the planting medium (vermiculite, perlite and soils at 1:1:1 ratio). All the plantlets survived (100%) and grew healthily in pots using this simple protocol. In contrast, Shrivastava and Kant (2011) reported a protocol which required the rooted *P. pinnata* plantlets to be grown in vermiculite moistened with half-strength MS medium before transferring to greenhouse. Various types of planting media formulated using soil, cow dung, vermiculite, vermiculture (Azotobactor), and sand (Belide et al. 2010; Sugla et al. 2007; Sujatha and Hazra 2007; Sujatha et al. 2008; Vasu et al. 2014) were attempted to acclimatise *P. pinnata* with approximately 56.6–98.0% of plant survival after 30 days transferring into these planting media. However, these studies did not specify the total number of plantlets used in their studies. In contrast, a simpler acclimatisation protocol with a much higher survival (100%; 30 plantlets) was achieved without application of vermiculture or cow dung in this study.

The acclimatised regenerated plants showed no morphological differences with the plants grew in the field. No morphological abnormality was observed. However, the roots of in vitro regenerated *P. pinnata* plants formed nodules when they were compared to the seed germinated field plants. The nodulation on root of a legume plant will have positive effect on nitrogen fixation, resulting in effective plant growth (Hayat et al. 2010). Moreover, denser microroots or secondary roots were observed from the in vitro regenerated plants compared to the seed germinated plants. This might be due to the application of STS where it enhanced the growth of microroots or secondary roots (Klerk et al. 1999; Mora et al. 2012). Further investigation is required to investigate the variations of root morphologies and root nodule development between the seed germinated plants and acclimatised in vitro regenerated plantlets.

Conclusion

With the establishment of the protocol for multiple shoot bud induction, regeneration, rooting and acclimatisation, micropropagation of *P. pinnata* can be performed. A complete plant regeneration system for multiple shoot buds was developed in this study to facilitate genetic manipulation study through methods, such as *Agrobacterium* or biolistic gun.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

CS and HL conceived, designed and supervised the study. SN carried out the experiments, analysed and interpreted the results. SN, CS and HL prepared the manuscript. All authors have read and approved the manuscript.

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