Effects of Plant Growth Regulators on Adventitious Roots Induction from Different Explants of *Orthosiphon Stamineus*

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**ABSTRACT**

*Orthosiphon stamineus* is a popular herbal tea that well known to be effective in treatment of kidney stone, depression and gall bladder problems. In this study, adventitious roots were induced from different explants (leaf, root and stem) of *O. stamineus*. Murashige and Skoog (MS) medium supplemented with auxins; indole-acetic acid (IAA), indole-butyric acid (IBA) and naphthaleneacetic acid (NAA) each at the concentrations of 0, 1, 3, 5, and 7 mg/L were evaluated for their effects on adventitious root induction. Results obtained revealed that the best rooting ability was observed in leaf explants cultured on MS medium supplemented with 3 mg/L of IAA. In this treatment, $88.3 \pm 12.6\%$ of rooting, $11.9 \pm 4.0$ roots per explants and $0.060 \pm 0.018$ g of dry weight were recorded. Although IBA and NAA also managed to induce the adventitious roots from leaf explants, the rooting ability was relatively lower than IAA treatment. The highest rooting ability for both IBA and NAA was achieved in 5 mg/L with $0.016 \pm 0.012$ g and $0.036 \pm 0.024$ g of dry weight, respectively. Root and stem explants were less efficient in inducing adventitious roots although managed to form some degree of adventitious roots. Root explants showed the highest rooting in 3 mg/L of IBA with $38.9 \pm 34.7\%$ whilst stem explants rooted more efficiently in 5 mg/L of IBA.

**Key words:**

Introduction

Adventitious roots have been reported in both technical and non-technical terms. Esau (1977) stated that adventitious roots are roots that arise from the sites other than usual sites, such as roots originating from stems or leaves. On the other hand, Barlow (1986) defined adventitious roots as roots that rise on parts of the plant not originating from the embryonic root; that is, the roots arise from parts of the shoot. Histological investigation with other species has shown that root initiation occur near the cambial zone or the phloem parenchyma, for example in cuttings of apple (*Malus*) (De Klerk, 1995) and chestnut (*Ballester et al.*, 1999) and in cuttings of hazel (*Rodriguez et al.*, 1988). Adventitious root is considered as commercial interest, since vegetative propagation by cuttings can be an efficient method for producing large numbers of plants, and is thus an attractive technique for the plant propagation industry (*Ford et al.*, 2001). It was reported that adventitious root induction technique has been applied on economically important plant; *Taxus baccata*, since it contains taxanes or diterpene amides that are the source of a chemotherapeutic drug (taxol) used to treat cancer (*Rowinsy et al.*, 1990). Besides, mass production of isoflavonoids through adventitious root cultures of...
Iris germanica had been conducted by Tomoyoshi et al. (2005) who noticed that its adventitious root cultures accumulated a high concentration of isoflavones that capable to be utilized in medicinal production.

Orthosiphon stamineus, locally known as the “cat whiskers”, has many traditional claims. For instance, the leaves are used for preparation of diuretic tea, which have been reported to be active against kidney and bladder inflammation (Eisai Indonesia, 1995). It is conventionally propagated by cuttings and seeds and it was reported that a slow seed germination, limited and delayed rooting of the vegetative cuttings have slowed down the mass propagation of this plant (Lim et al., 2006). These problems caused a limited supply of O. stamineus plants in meeting the market demand especially in pharmaceutical and herbal industry. To overcome the shortage of raw materials, several approaches have been carried out by scientists. Cell suspension culture of O. stamineus that managed to produce rosmarinic acid and antioxidant compounds has been established successfully by Lee and Chan (2004). However Olah et al., (2003) stated that the roots of O. stamineus are expected to contain a higher amount of active compounds such as rosmarinic acid, polymethoxylated flavonoids and the caffeic acid derivatives than the organs like stem and leaf. Similarly in an earlier study, an important medicinal compound, ginsenosides has been largely produced from adventitious roots of Panax ginseng (Sivakumar et al., 2006). In addition, tropane alkaloids have been produced from the hairy and adventitious root cultures of Duboisia myoporoides-D, Leichhardtii hybrid (Yoshimatsu et al., 2004).

Although many studies on O. stamineus have been conducted, the study on adventitious roots of O. stamineus is still very limited. In view of the importance of adventitious roots as well as to further explore the potential of adventitious roots of O. stamineus, thus the present study was carried out to determine the effect of different auxins at various concentrations on the adventitious root induction from different explants of O. stamineus as well as to identify the most suitable explants of O. stamineus for adventitious root induction.

Materials and methods

Plant Materials:

In vitro plantlets of Orthosiphon stamineus that cultured on basal Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) were used as plant materials in this study. Only young and fresh leaf, root, and stem explants were selected throughout the experiment.

Culture Medium:

Full strength of MS medium was used in this experiment. The medium was prepared from stock solutions that the medium consists of macro- and micronutrients, MS vitamins and Fe-EDTA as in Appendix A. Auxins (IAA, IBA and NAA) (MERCK, Germany) as plant growth regulator (PGR) were added singly to the media. A total of 5 % of sucrose (w/v) was added into the media as the carbon source. The pH of the medium was adjusted to 5.8 ± 0.1 with 0.1M HCl or 0.1M NaCl before 0.8 % of agar powder (w/v) (Laboratory Reagents) was added. The medium was then autoclaved at 121 °C and 15 Psi for 15 minutes. Finally, the autoclaved media were poured into sterile Petri dishes under laminar flow.

Effects of Different Auxins at Various Concentrations on Different Explants:

To investigation the effects of different auxins on adventitious roots induction in O. stamineus, 0.5 cm x 0.5 cm leaf segments, 0.5 cm long root and stem explants were excised from intact in vitro plantlets. The study on leaf explants was initiated by placing the abaxial (lower) surface of the explants facing the medium. Meanwhile, root and stem explants were placed in a way that the whole explants in contact with the medium. Three different auxins (IAA, IBA and NAA) at the concentrations of 1, 3, 5 and 7 mg/L were used in this experiment. The MS medium which was PGR free was used as the control medium in each treatment. All the cultures were maintained in a culture room and allowed to grow at 25 ± 1 °C. The cultures were also kept in 24 hours dark condition throughout the 4 weeks culture period. For each different treatment, 5 explants were cultured on each Petri dish and 3 replicates were prepared. All the treatments were also repeated twice.

The initiation of root from explants was recorded on the basis of visual observations. Results were expressed as mean ± standard deviation. Observation on explants, the rooting percentage (%), dry weight (g), number of roots formed per explant and any morphology changes were recorded continuously for 4 weeks of the interval of every 2-3 days after cultured. As for the dry weight, the adventitious root induced in each treatment were excised and collected after 4 weeks of culture. Prior to the blotting of the roots, the weight of the filter paper was determined and recorded. The roots were then collected on filter paper, and allowed air
dry for 15 minutes. In order to measure the dry weight, the roots collected were oven-dried at mild temperature until a constant weight was obtained.

Statistical Analysis:

All data collected from each treatment were statistically analysed using One Way Analysis of Variance (one-way ANOVA). Tukey’s HSD test with the mean comparison was made by at least significant differences at the 5% probability level (P < 0.05). All the statistical analysis was conducted using the statistical software of SPSS (version 12.0).

Results and discussion

The Best Explants in Adventitious Root Formation:

Leaf explants was found to be the best explants of Orthosiphon stamineus in adventitious root induction. This was verified in terms of better percentage of rooting, number of root formed per explants and dry weight of induced roots obtained. MS medium supplemented with IAA, IBA and NAA regardless of its concentration gave rise to a higher amount of roots formed from leaf explants. In average leaf explants managed to obtain the highest number of roots per explants (11.9 ± 4.1) which brought to 0.060 ± 0.018 g of dry weight (Figure 1). Meanwhile, lower rooting efficiency from root explants was supported with lower number of roots per explants (1.1 ± 1.1), and no dry weight of induced roots was obtained from roots explants (Figure 2). Meanwhile, stem explants also showed lower rooting efficiency with relatively lower number of roots induced per explants (2.53 ± 1.70) and dry weight (0.01 ± 0.01 g) as compared to leaf explants (Figure 3).

The positive effect of leaf explants in adventitious root formation have been demonstrated in earlier histological studies of root formation from leaf explants of Medicago truncatula (Rose et al., 2006). A higher efficiency of leaf explants in rooting was also observed on Cycads with a higher tendency to develop roots from leaf bases (Pant, 1990). In addition, observation by Osborne (1994) in Africa showed that Encephalarios leaf bases rooted easily, even without treated by rooting hormones. It was proven that leaf explants was effective in inducing morphogenic response in tissue culture studies of other plant species, such as Lycopersicon eculentum Mill (Sheej et al., 2004) and potato (Yasmin et al., 2003) in plant regeneration protocol (rooting and shooting).

Leaf segments acted as better explants in rooting might due to the presence of cells associated with the leaf veins, which can be readily stimulated by adding auxins. Thus, the use of leaf explants in tissue culture has the advantages of being a system whereby phytohormones can easily be manipulated to direct pluripotent cells to a particular cell fate (Imin et al., 2005; Thomas et al., 2004; Nolan et al., 2003; Schmidt et al., 1997). These vein-associated cells were stimulated to divide in response to auxins and grow distinctive sheets cells that origin from the veins of the leaf explants. This is important as these procambial-like cells, which function as pluripotent stem cells can be switched into forming either roots or somatic embryos depending on the prevailing hormone status of the media (Rose et al., 2006).

In addition, leaf explants found to be more effectively in giving rise to the adventitious roots than stem and root explants could also be due to its ability to produce endogenous auxins that was essential for root initiation (Eduardo, 1998). The endogenous auxin produced on the apical site of plants will be transported downward to the basal part of plants like stem and root site in a polar manner (Terasaka et al., 2005). However, earlier study has reported that root and shoots also producing IAA (Nordstrom et al., 2004; Ljung et al., 2005). The ability of leaf explants to produce endogenous IAA may resulted in the leaf explants become less dependent on exogenous IAA supplied from culture medium. The similar results was shown in Luffa cylindrica, whereby its leaves at the proximity between the sites of IAA production and the sites of differentiating vascular cells probably resulted in relatively high local IAA concentration at the differentiating sites (Aloni, 2001). This could also possibly explained why leaves can readily rooted in rooting medium (Horner et al., 1994).

The Best Auxin for Adventitious Root Formation:

The re-establishment of the rooting system from tissue without pre-existing meristems is often strictly dependent on the application of exogenous auxin (Ricci et al., 2004). The important role of auxins on rooting has also been previously reported by Pan and Tian (1999). Other than that, earlier studies have shown that the auxins treatments either stimulated or inhibited root production depending on the concentration used (Eduardo, 1998). Thus, in this experiment only auxin (IAA, IBA and NAA) were selected to induce adventitious root from O. stamineus.
Fig. 1: Effects of the addition of IAA at various concentrations on the adventitious roots induction from in vitro leaf explants of *O. stamineus* incubated in dark condition after 21 days of culture. (a) 0 mg/L (b) 1 mg/L (c) 3 mg/L (d) 5 mg/L (e) 7 mg/L

Fig. 2: Effects of the addition of IAA at various concentrations on the adventitious roots induction from in vitro root explants of *O. stamineus* incubated in dark condition after 28 days of culture. (a) 0 mg/L (b) 1 mg/L (c) 3 mg/L (d) 5 mg/L (e) 7 mg/L
Fig. 3: Effects of the addition of IAA at various concentrations on the adventitious roots induction from in vitro stem explants of *O. stamineus* incubated in dark condition after 25 days of culture. (a) 0 mg/L (b) 1 mg/L (c) 3 mg/L (d) 5 mg/L (e) 7 mg/L.

Fig. 4: Effects of the addition of NAA at various concentrations on the adventitious roots induction from in vitro leaf explants of *O. stamineus* incubated in dark condition after 21 days of culture. (a) 0 mg/L (b) 1 mg/L (c) 3 mg/L (d) 5 mg/L (e) 7 mg/L.

An overall comparison of three different auxins tested (IAA, IBA and NAA) at various concentrations (0, 1, 3, 5 and 7 mg/L), showed that IAA was the best auxin for *O. stamineus* in adventitious roots formation. IAA naturally was synthesized in the apical part of plants like leaves and shoot apices (Terasaka *et al*., 2005). This endogenous IAA then will be transported downward to lower part of the plants in polar manner until
reaching the rooting zone (Booker et al., 2003). Auxin IAA also reported to induce dedifferentiation of cells in the rooting zone that may be necessary for subsequent root formation during subsequent days of rooting culture (Ammirato 1985). The positive effect of IAA in rooting was due to its crucial role in many aspects of root growth, development and differentiation (Aloni et al., 2003). It was also reported that IAA is responsible for regulating the development of the primary and lateral, root apical meristem and root vascular differentiation (Sachs, 1981; Ponce et al., 2005). Adventitious root induction in response to IAA was due to the interruption of IAA transport in the bundle sheath of leaf cuttings, whereas xylem regeneration is induced by the disturbance of IAA movement through the vascular meristem (Aloni, 1987). IAA accumulation in the IAA transporting tissue (phloem) can induce the initiation of adventitious roots (Aloni et al., 2004). In contrast, other plant species like *Backhousia citriodora* showed different response to auxin, because a lack of endogenous auxins did not limit the formation of adventitious roots in cuttings (Davies, 1984). As the best auxin in adventitious root formation from leaf explants of *O. stamineus*, IAA however gave different results in rooting efficiency when different concentrations were applied. It was showed that besides the types of auxins used, the particular concentration was also crucial in inducing rooting. As shown in this study, MS medium supplemented with IAA at 3 mg/L showed better rooting response than other concentrations tested, whereas an increase in concentration from 3 to 5 mg/L or a decrease from 3 to 1 mg/L decreased the capacity of rooting from leaf explants. It was noticed that at 3 mg/L, it gave rise to the highest rooting ability in terms of number of roots induced per explant (11.9 ± 4.1) and dry weight of roots (0.060 ± 0.018 g). Lower rooting ability at low IAA concentration (1 mg/L) may due to inadequate supply of IAA that was necessary in roots induction from leaf explants. During root differentiation, a relatively higher IAA concentration was required and subsequently induced cell division and root initiation (Aloni et al., 1980). This could explain why rooting efficiency was better in 3 mg/L of IAA as compared to lower concentration (1 and 2 mg/L). Similar result was reported by Visser et al. (1996) whereby a decrease in IAA caused reduction in the number of adventitious roots formation by flooded plants.

Meanwhile, higher concentration (5 mg/L and 7 mg/L) have also showed a lower rooting efficiency. This could be explained by the fact that auxins at high concentration may possess herbicidal property which may inhibit the adventitious root induction from leaf explants (Evan et al., 2003). Besides, an overdose of auxins in medium will actually inhibit cell elongation, because the plant will produce other hormone to balance the act. Furthermore, high concentration of auxins will also inhibit the root growth and development and resulting in root apical dormancy (Chao et al., 2006). Syros et al. (2004) and Rout et al. (2000) demonstrated that the treatment with a higher concentration than the optimum concentration of auxins inhibited the rooting of *Psoralea corylifolia* and *Ebenus cretica* cuttings, respectively.

IBA and NAA are synthetic auxins that also found to be successfully induced adventitious root from leaf explants, with a lower percentage as compared to IAA. Both synthetic auxins (IBA and NAA) found to show better rooting ability at the same concentration (5 mg/L). In both IBA and NAA treatments, a trend of increase in rooting efficiency was recorded in response to increase in IBA and NAA concentrations from 1 mg/L to 5 mg/L. Study also suggested that 5 mg/L was the optimum concentration for IBA and NAA, however further increase to 7 mg/L of IBA and NAA caused a decrease in rooting ability. In 5 mg/L of IBA, 6.3 ± 3.8 of roots was induced and 0.016 ± 0.012 g of dry weight was recorded. On the other hand, 6.6 ± 0.6 roots per explants and 0.038 ± 0.016g of dry weight were recorded in medium supplemented with 5 mg/L of NAA (Figure 4). The reasons of lower rooting efficiency from IBA and NAA treatments might be due to factors like species dependent, in which different species responded to same type of plant growth regulator differently (Wei et al., 2006). Some plants species were reported to response better in IBA or NAA treatments. For example, better rooting was discovered in the IBA treatment for Ginseng (Choi et al., 1994), meanwhile NAA treatment was found to be a better auxin for Mung bean (Karim et al., 2005).

The lower efficiency of IBA and NAA in adventitious root induction could also be explained by the fact of the relationship between levels of endogenous IAA and adventitious root formation. It was suggested that the exogenously applied synthetic auxins (IBA or NAA) have not been efficiently oxidized to IAA for plant cell utilization. Thus, in the condition of insufficient supply of IAA, the explants will show lower ability in rooting initiation. This statement was supported by an earlier study which stated that endogenous IAA was detected in root explants of both IBA and NAA supplemented treatments (Liu et al., 1988). Accumulation of endogenous IAA in soybean hypocotyl explants was found during adventitious root formation in NAA and IBA treatments. Thus, it was suggested that the exogenously applied auxins (IBA and NAA) may act on IAA oxidase enzyme activity during the formation of adventitious roots in soybean hypocotyls (Ludwig et al., 1991). IBA has been shown to be converted to IAA by several plant species (Baraldi et al., 1993). Furthermore, the explants in the culture may use up more energy when converting the absorbed synthetic auxins (IBA and NAA) from medium to natural form of IAA before been used by the explants. This could possibly explain the lower efficiency in rooting induction of IBA and NAA. As a result, more energy will be used up and subsequently may lead to insufficient energy for cell growth and development during root formation.
It was reported that high concentration of both IBA and NAA (7 mg/L) induced lower degree of rooting which may be due to the herbicidal activity of auxins at high concentration as stated by (Evan et al., 2003). Meanwhile, lower concentration was insufficient to stimulate the best rooting induction. Particularly for NAA, Hauman (1993) demonstrated that certain plant tissue were relatively low in absorption of NAA, thus it may resulted in insufficient supply of auxin for leaf explants to induce rooting.

It has been widely reported that plant cell cultures such as callus cell suspension have a strong tendency to be genetically and biochemically unstable and often synthesize very slow levels of useful secondary metabolites. Adventitious root culture can be an attractive alternative as it is highly differentiated and can cause stable and extensive production of secondary metabolites. Thus, further studies can be carried out by focusing on the identification and quantification of some important medicinal compounds such as rosmarinic acid, oxygenated diterpenes and sinensitin that present in the adventitious roots using the liquid medium. The application of liquid medium in induction of adventitious root formation can further facilitate the scaling-up of root cultures in the bioreactor system for large scale and commercial production (Alvard et al., 1993).

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References


