INDUCE REGENERATION IN IN VITRO CULTURES OF AGARWOOD PLANT SPECIES, Aquilaria filaria (INDUKSI REGENERASI *IN VITRO* PADA SPESIES TANAMAN PENGHASIL GUBAL GAHARU, *Aquilaria filaria*)

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ABSTRACT

Aquilaria filaria is one of the species producing precious fragrant oleoresin agarwood which is endemic in Eastern of Indonesia. In 2004 CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) incorporated *A. filaria* as vulnerable species due to the declining population of this plant in their habitat of origin. Propagation of this plant species is essential for continuation of agarwood supply and preservation of genetic resources. Biotechnological approach by plant *in vitro* culture is advanced preservation technique for genetic resources. Plant *in vitro* culture is not only strategy to propagate plant material in prolific rates for in situ and ex situ germplasm preservation in range of environmental condition, but also answer the problem in seed recalcitrant and less viability as well as studying genetic and physiology of the plant. Up to date there is no available information of *in vitro* regeneration in *Aquilaria filaria* plant. This research aims to study de novo regeneration of shoot and root in plant tissue culture of *Aquilaria filaria* for the basis of *in vitro* preservation of this vulnerable plant species. The research includes callus induction, shoot regeneration from plant callus and root regeneration from shoot explants. In vitro cultivation conducted in Murashige and Skoog (MS) medium containing phytohormone auxin: indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) and cytokinin: 6-benzylaminopurine (BAP) and kinetin. The result reveals that leaf is competent material for callus induction. High proliferating callus is induced in medium containing combination of IBA (2 mg/l) or NAA (0.2 – 1 mg/l) with cytokinin BAP (0.5 – 1 mg/l). *A. filaria* plant callus regenerate shoots primordia by an addition of single BAP (0.5 mg/l) or combination with IBA or NAA (0.2 mg/l)) into the culture medium. Moreover, rooting of shoot cultures achieve in medium with additional of sole phytohormone IBA (1 – 2 mg/l) or NAA (0.2 – 2 mg/l). The result of this study provides basis for *in vitro* propagation of *A. filaria* and further assessment related agarwood producing species.

Key words: *Aquilaria filaria*, Murashige and Skoog (MS) medium, indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP) and kinetin.

ABSTRAK

Aquilaria filaria adalah salah satu spesies penghasil gaharu, gubal wangi bergengsi tinggi, yang endemik di Indonesia bagian timur. Pada tahun 2004 CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) memasukkan *A. filaria* sebagai spesies yang langka karena penurunan populasi dari tanaman ini pada habitat aslinya. Oleh karena itu, perbanyakan dari spesies tanaman ini sangat penting untuk keberlanjutan suplai gaharu dan pelestarian sumber genetik. Pendekatan biotechnologi melalui kultur *in vitro* tanaman adalah teknik terdepan untuk pelestarian sumber genetik. Budidaya tanaman *in vitro* tidak hanya strategi untuk memperbanyak tanaman secara masal untuk pelestarian sumber genetik pada kondisi linggungan yang luas, tetapi juga menjawab permasalahan benih rekalsitran dan viabilitas rendah, selain juga untuk mempelajari genetik dan fisiologi dari tanaman tersebut. Hingga saat ini belum ada informasi yang tersedia mengenai regenerasi *in vitro* pada tanaman *Aquilaria filaria*. Penelitian ini bertujuan untuk mempelajari de novo (permulaan) regenerasi pucuk dan akar pada kultur jaringan tanaman *Aquilaria filaria* sebagai basis pelestarian *in vitro* dari spesies tanaman ini. Penelitian ini termasuk induksi kalus tanaman, regenerasi pucuk dari kalus tanaman dan regenerasi akar dari eksplan pucuk. Kultur *in vitro* dilakukan menggunakan medium Murashige and Skoog (MS) yang mengandung phytohormone auxin: indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) dan cytokinin: 6-benzylaminopurine (BAP) and kinetin. Hasil penelitian ini menunjukkan bahwa

daun adalah material yang kompeten untuk induksi kalus. Proliferasi kalus yang tinggi terinduksi pada medium yang mengandung kombinasi IBA (2 mg/l) atau NAA (0.2 – 1 mg/l) dengan cytokinin BAP (0.5 – 1 mg/l). Kalus tanaman *A. filaria* beregenerasi menjadi pucuk primordial dengan penambahan BAP (0.5 mg/l) atau kombinasi IBA atau NAA (0.2 mg/l)) kedalam medium kultur. Selanjutnya pengakaran dari kultur pucuk didapatkan pada medium dengan hanya penambahan IBA (1 – 2 mg/l) atau NAA (0.2 – 2 mg/l). Hasil penelitian ini memberikan basis bagi propagasi *in vitro A. filaria* dan penelitian lanjutan bagi spesies tanaman penghasil gaharu.

Kata kunci: Aquilaria filaria, *medium Murashige dan Skoog (MS), indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP) dan kinetin.*

INTRODUCTION

The plant genus of *Aquilaria* are well known for their capability to produce agarwood, a priceless non-timber forest commodity from South East Asia. Agarwood is fragrant oleoresin formed inside the wood of the tree (Jensen, 2009). Beside its traditional application as medicine and element in religious ceremony of Asian and Middle Eastern countries, agarwood oleoresin is an important material for modern perfumery industry including leading brand company (Akter et al., 2013). From the ancient time up to date, agarwood remains as an economical commodity exported from Southeast Asian to Far East, Middle East and European countries as well as USA (Antonopoulou, et al., 2010; Wyn and Anak, 2010). Recently, the price over the top grade of agarwood oil is exceeding USD 30.000 in a kilogram (Government of India Ministry of Environment and Forests, 2014).

Aquilaria filaria ecological niche is in low land forest in the island of Celebes, Moluccas and West Papua, at the eastern region of the country Indonesia. *A. filaria* is one of the major resources of agarwood exported from Indonesia along with *A. malacensis* which distributes in western region (Patmomihardjo and Semiadi, 2008). In fact Indonesia is the largest exporting countries of agarwood, reach 80% of the global supply (Jensen, 2009). However, the tendency of agarwood export quota from *A. filaria* derivation is decline from 250 ton in 1999 to 60 ton in 2008 as the decrease number of population in natural habitat (Samedi, 2006; Patmomihardjo and Semiadi, 2008). One reason of rapid depletion of *A. filaria* in nature is a consequence of random logging from the past to harvest the oleoresin content without considering the plant living period. Long maturation time is necessary for the formation of refined agarwood in nature. High grade agarwood is likely to be found in 50 years old of tree (Damania, 2001). Therefore the government of Indonesia endorse the incorporation of *A. filaria* as vulnerable species and included into the Appendix II of CITES (CITES, 2004).

As included in Appendix II of CITES, in situ and ex situ preservation of *A. filaria* is outlawed to be put off. Plant preservation via seed propagation stands with problems of seasonal production, seed recalcitrant or short time of viability, seed deterioration and pathogen bearing seed (Johnson, 2002; Belokurova, 2010). Biotechnological approaches by plant tissue cultures award the technique for logarithmic number of regeneration within short period of time by small pieces of plant (explant) which difficult to be obtained by seed propagation (Rai, 2010). In addition, plant in vitro cultures is beneficial for reintroduction, study, demonstration and commercial application (Pence, 2011).

Su and Zang (2014) reveal that phytohormone is the main regulator in plant regeneration. It is also disclose the two method of *in vitro* plant regeneration by direct and indirect regeneration. Direct regeneration is when organ regenerated directly from plant tissue while indirect regeneration is organogenesis from induced plant callus. Plant callus defined by meristematic cells similar to the root tip, regarding it induce from explants of basal or aerial organ of the plant (Sugimoto et al., 2010). Regeneration via callus culture is also important for further biotechnological application of the plant.

Up to date there is no available information of *in vitro* propagation of *A. filaria*, nor indirect shoot and root regeneration from callus cultures of this agarwood producing species. This research aims to study regeneration shoot and root of in vitro cultures of *A. filaria* by direct and indirect methods.

MATERIALS AND METHODS

Plant Materials

Aquilaria filaria sterile in vitro seedlings were obtained from BPPT, Serpong, Indonesia. *A. filaria* mature fruit, yellowish color, were surface sterilized with sodium hypochlorite (NaOCl) 3% and rinse with steril water for three times. The fruits were opened and five of seeds were transferred into 25 ml Murashige and Skoog (MS) agar medium without additional phytohormone (MS-0). *In vitro* seed cultures were incubated at 27^{o}C under continuous light until the plantlet had 3-5 leaves. Plantlet leaves were used as plant explants material for *in vitro Aquilaria filaria* callus, shoot and root induction.

Callus Induction

Induction of callus from *A. filaria* aimed to produce callus from plant part which will be used for vegetative regeneration of *A. filaria* shoots or roots. Callus regeneration was initiated from approximately 0.5 cm^2 of cut-leaves materials. The leaves explants were overlaid in 90 mm petridishes containing 25 ml of MS agar medium supplemented by 20 g/l of sucrose and phytohormone auxin, cytokinin or combination of both. Auxin type used was indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) in the range of concentration 0.2 – 2 mg/l. Cytokinin types used were 6-benzylaminopurine (BAP) and kinetin in concentration of 0.5 – 1 mg/l. Each petridish contained 5 leaves explants. Control treatment was made without supplementation of phytohormone into MS medium. All off the treatments were made in triplicate. The plant cultures were cultivated for 4 weeks at 27^{o}C in dark. Control cultures made in medium without additional phytohormones. Fresh callus were weighted after harvesting.

Shoot Regeneration from Callus Cultures

This experiment was aim to induce shoot from friable callus of *A. filaria in vitro* cultures. Callus was prepared from 0.5 cm^2 of leaf explants as done in callus induction section. Callus from leaves explants were cultivated in MS medium supplemented by 20 g/l of sucrose and single phytohormone cytokinin BAP (0.5 mg/l) or combination with auxin, IBA or NAA (0.2 – 2 mg/l). The cultures were incubated without light at 27^{o}C for 4 weeks for callus induction then followed by 4 weeks cultivation in continuous light at similar temperature. Control cultures made in medium without additional phytohormones. Then, the regeneration of callus was observed.

Root Regeneration from Shoot Cultures

Root induction was done from *A. filaria* shoot with aims to have complete in vitro plant seedling which ready for acclimatization. Approximately 3 - 4 cm of in vitro *A. filaria* shoot was used as explant materials. In this experiment single phytohormone NAA and IBA in range concentration of 0.2 – 2 mg/l were used to induce root formation. The explants were cultivated for 4 weeks in growth chamber at 27^{o}C with continuous illumination then the formed root were evaluated. Control cultures made in medium without additional phytohormones.

RESULTS AND DISCUSSION

Results

Callus Induction

Calli grown from *A. filaria* leaves explants cultivated in dark. Calli observed start to emerge within one week of cultivation. The growth of callus was observed in all of the treatments applied, either in medium with single auxin, cytokinin or combination of both (Fig. 1A). However, types, combination and concentration of phytohormones added into the culture medium affect callus proliferation. After 4 weeks of cultivation there are differences in callus fresh weight obtained from various treatments applied (Fig. 1B).

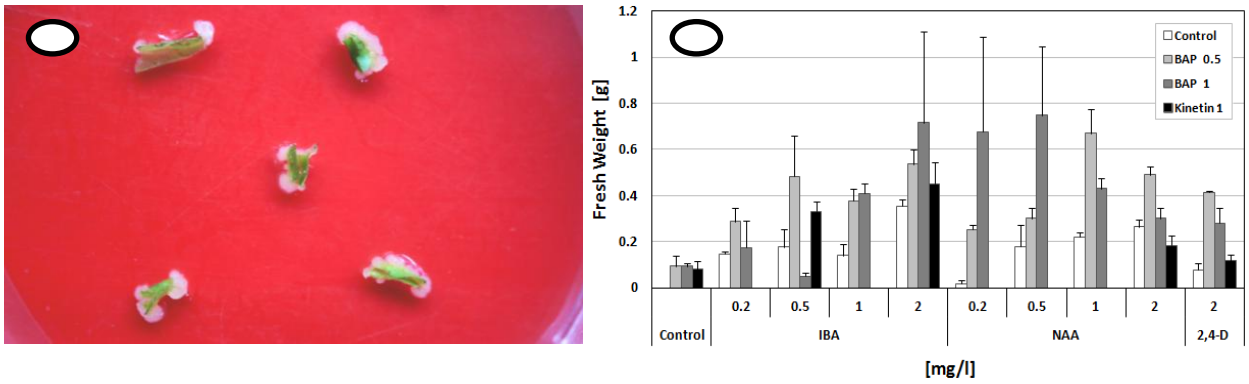

Fig. 1: (A): Calli grow from *A. filaria* leaves explants cultivated in medium containing phytohormones, auxin and cytokinin. (B): Fresh weight of callus obtained from 4 weeks cultivation of *A. filaria* leaves explants in MS medium supplemented by phytohormone auxin and cytokinin.

In general, application of single auxin or cytokinin induces less of fresh callus weight compared to combination of both. Auxin type IBA and NAA showed to induce higher callus proliferation than 2,4-D. In addition, cytokinin BAP performs well than Kinetin. Moreover, the highest fresh weight of callus obtained from leaves explants grown in medium supplemented by combination of auxin IBA or NAA with cytokinin BAP. IBA shows to induce more calli in higher concentration (2 mg/l) while NAA effective in lower concentration (0.2 – 1 mg/l). In addition, combination of those concentrations of IBA or NAA with 0.5 – 1 mg/l of BAP promoted more of the callus growth. Up to 4 weeks of cultivation, the amount of fresh callus weight obtained from combination of (NAA 0.5 mg/l + 1 mg/l BAP), (IBA 2 mg/l + mg/l BAP), (NAA 0.2 mg/l + 1 mg/l BAP) and (NAA 1 mg/l + 0.5 mg/l BAP) were 0.75g, 0.72g, 0.68g and 0.67g respectively. Callus induction responds was 100% in almost all treatments applied.

Shoot and Root Regeneration

The successful of mass propagation by plant tissue culture rely on the capability of plant callus to differentiate into shoot and root. Four weeks dark-cultivated leaf explants with growing callus were subjected to illumination for stimulation of shoot regeneration. Leaf callus cultivated in lower ratio of auxin to cytokinin, from white callus in dark cultivation develop to become green in light cultivation (Fig. 4A). Callus in higher ratio of auxin to cytokinin in the beginning of culture under illumination turn from white callus to become yellow (Fig. 4B), however gradually change to become green. After 6 weeks of cultivation under illumination there is development of nodular embryonic structures (NES) which served as shoot primordia from leaves callus. NES were observed in treatment medium of single cytokinin, BAP (0.5 mg/l) and combination with auxin, IBA or NAA (0.2 – 2 mg/l). In general IBA or NAA have no different effect in the number of NES observed, however the concentration of each phytohormone influence regeneration of NES from callus. The highest number NES reach in medium containing (BAP 0.5mg/l + 0.5mg/l NAA) and (BAP 0.5mg/l + 0.2mg/l BAP) with average number of NES per explant counted for 6.4 and 5.8, respectively. There are two types of NES observed regenerate from leaf callus, translucent node type (Fig. 4C) and compact node type (Fig. 4D).

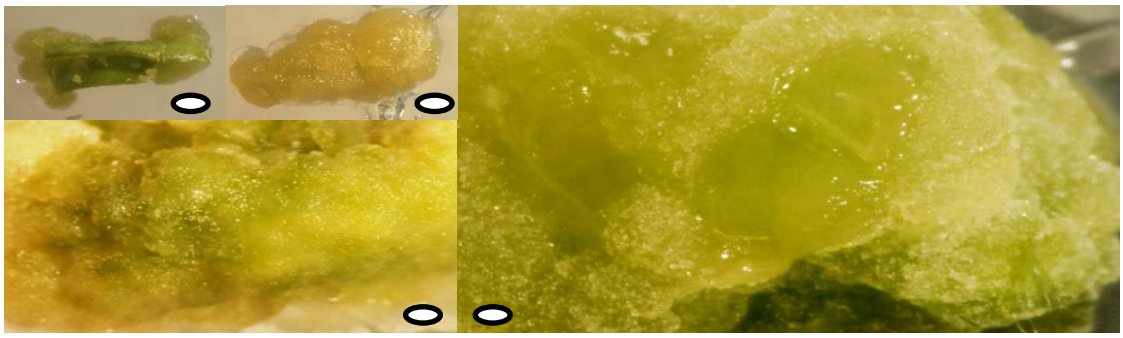

Fig. 4: Regeneration of *A. filaria* callus from leaves explants. (A): Higher cytokinin than auxin, callus turn green. (B): Cytokinin lower than auxin callus turns golden yellow. (C): Translucent type of Nodular Embryonic Structure (NES). (D): Compact type of NES.

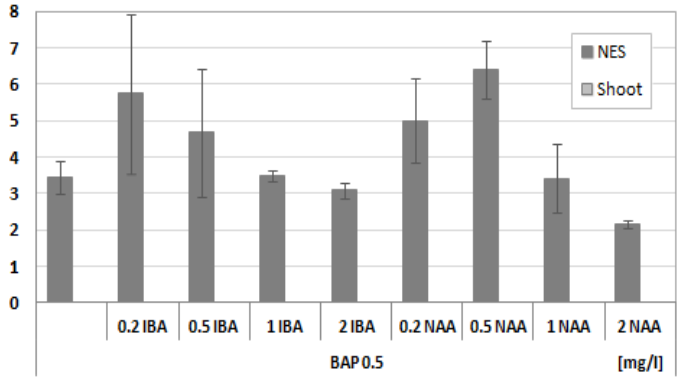

Fig. 5: Number of Nodular embryonic structure (NES) per explants regenerate from callus derived leaf after 6 weeks of cultivation under illumination.

In order to have complete plantlet organogenesis, shoot explants of *A. filaria* were stimulated for rooting. It can be seen from the graph (Fig. 5D) that NAA or IBA were competent to induce rooting from *A. filaria* shoot explants. However, roots regeneration from shoot cultures show to be most effective in higher concentration of NAA. After 4 weeks of cultivation, application of 2 mg/l of NAA induced on average of more than 2.5 of healthy roots per explants with responds frequency of 100%.

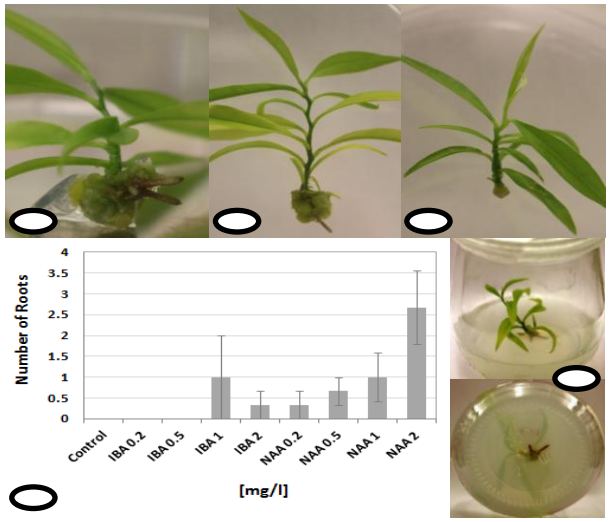

Fig. 6: Root regeneration from shoot cultures of *A. filaria*.
(A, B, E): Roots grow from shoot explants of *A. filaria* after 4 weeks of cultivation in MS medium containing auxin (NAA or IBA) 0.5 – 2 mg/l. (C): No root grows from control treatment without addition of phytohormone. (D):
The number of root emerge from shoot cultures of *A. filaria* after 4 weeks of cultivation in MS medium containing auxin (NAA or IBA) 0.5 – 2 mg/l (below)

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Discussion

Development of efficient method in *in vitro* plant regeneration is one strategy for mass propagation of plant species. This article describes *in vitro* shoot and root regeneration of *Aquilaria filaria* as a basis method for in vitro propagation of this plant vulnerable species.

Callus Induction

Some papers have illustrated the method of callus induction from leaves or nodal explants of some Aquilaria species, those are *A. sinensis* from Taiwan, *A. crassna* from Thailand and Vietnam, and *A. malacensis Lam.* and *A. agallocha Roxb*. from India by phytohormone auxin, 2,4-D or NAA, and cytokinin BAP. Moreover, it was investigated that Murashige and Skoog (MS) medium was better than Woody Plant Medium (WPM) for cultivation of callus (Okudera and Ito, 2009; Saika et al., 2012; Saika et al. 2013; Debnath, 2013). Alike to this research, calli proliferate from leaves explants of *A. filaria* in all hormone-treatment medium (fig. 1). Additionally, high fresh weight of callus obtained from explant cultivated in MS medium supplied by auxin type IBA (2 mg/l) or NAA (0.2 – 1 mg/l) combined with cytokinin BAP (0.5 – 1 mg/l).

In vitro callus proliferation is controlled by auxin-rich callus induction media (CIM) in synergistic effect with an addition of cytokinin (Ikeuchi et al., 2013). In regard to this study, application of auxin and cytokinin in combination into growth medium produce callus more than of single phytohormone. In addition, *A. filaria* callus emerge in higher amount in sole medium of auxin compared to medium with cytokinin individually. High callus fresh weight obtained in this study by ratio of auxin IBA (2 mg/l) to cytokinin BAP (1 mg/l) is also in agreement to previous studies. Even though in application of lesser auxin ratio to cytokinin, NAA (0.2 – 0.5 mg/l) to BAP (1 mg/l), high callus fresh weight is also achieved. The reasons might be due to the influence of endogenous phytohormone within explants, type of auxin and cytokinin and source of explants (Jimenez and Bangerth, 2001; Yancheva et al., 2003; Welander and Snygg, 1987).

Shoot and Root Regeneration in Shoot Cultures

In general, cytokinin-rich shoot induction medium (SIM) is used for shoot induction (Motte, 2014). In consistence to this research, medium with single BAP (0.5 mg/l) and combination with less auxin NAA or IBA (0.2 mg/l) promote development of shoot primordia from leaf callus. Debnath et al. (2013) are also found that 0.2 mg/l BAP induce 18 fold of shoot buds from nodal segment and shoot tip of *A. agallocha*.

In high ratio of cytokinin to auxin white callus turn green in light cultivation, whilst in higher ratio of auxin than cytokinin (2 : 1) yellow callus observed which finally become necrotic. Kobayashi et al. (2012) explain by studying detach root of Arabidopsis that cytokinin has positive regulation to chlorophyll biosynthesis and chloroplast biogenesis conversely to auxin which promote negative regulation. However, beside its negative effect, certain amount of auxin is needed for shoot regeneration. Furthermore it has been proved that cross talk between auxin and cytokinin occur during de novo shoot regeneration development (Kakani and Peng, 2011; Cheng et al., 2013; Su and Zhang, 2014).

Root induction from shoot cultures is achieved in medium containing single auxin. Different from regeneration of root in callus culture, root regeneration from shoot cultures of *A. filaria* is more effective induced by NAA (0.2 – 2 mg/l) compared to IBA (1 – 2 mg/l). He et al. (2005) shows similar result in stimulation of rooting from shoot of *A. agallocha* by NAA. However, stimulation of root regeneration from *Aquilaria crassna* shoot culture shows IBA more effective in root regeneration than NAA, while no root grows from *A. malacensis* shoot cultures (Tiengtum, 1995). It is suggested that different species of Aquilaria giving specific response to certain auxin type.

CONCLUSION

In situ and ex situ preservation of *A. filaria* requires appropriate method for propagation. The results from this research provide basic method for in vitro micropropagation of *A. filaria*. In vitro regeneration of A. *filaria* can be done from the induction of callus culture followed by regeneration

of shoot from callus and root from shoot culture. Calli induce from leaf explants in auxin rich medium combined with low or equal concentration of cytokinin. Conversely, medium for shoot induction is sole cytokinin or combine with lower concentration of auxin. Rooting from shoot culture only perform in medium containing single auxin. More over our finding in rooting of callus provides also possibility to induce shoot regeneration prior to stimulation of shoot.

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