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Rapid Induction of Somatic Embryos and Production of Synthetic Seeds from Hempedu Bumi (*Andrographis paniculata*) - A Malay Ethnomedicinal Plant

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Abstract

Hempedu Bumi, *Andrographis paniculata* a popular Malay ethnomedicinal plant used to treat various kinds of diseases like common cold, fever, liver disease and snake bite. Andrographolide a bitter compound present in the plant posses many medicinal properties like anti-inflammatory, antiviral, anti-HIV and promising anticancer activity. Large amount of the compound is being extracted from the plants so there is a need for commercial cultivation of this plant, but seed dormancy proved to be a major obstacle for this process. So production of non-dormant synthetic seeds is an alternative to the dormant natural seeds for the mass cultivation of this plant. Hence the present study is aimed to produce somatic embryos and synthetic seeds using plant tissue culture technique. The zygotic embryos from immature seeds of *A. paniculata* were inoculated on to MS media containing various auxins (NAA, IBA, 2, 4-D), and cytokinins (BAP, TDZ and Kinetin) for the induction of somatic embryos. Out of all the treatments used, only 1.5 mg/L NAA + 0.75 mg/L Kinetin produced somatic embryos after 6 weeks of culture and the number of somatic embryos per explant was 9.8 ± 0.6. NAA alone produced rhizogenic calli and NAA along with BAP produced hard friable white callus, 2, 4-D alone and 2, 4-D with cytokinins (BAP, Kinetin) produced creamy callus, while IBA alone and in combination with cytokinins (BAP, Kinetin), TDZ resulted in germination of embryos into seedlings. The somatic embryos produced were coated with sodium alginate solution and made into synthetic seeds. The synthetic seeds that have been produced can be used as planting material and for conservation of the plant for future uses.

1. Introduction

Andrographis paniculata Nees. belongs to genus Andrographis, family Acanthaceae is a popular ethno-medicinal herb used for treating infection, inflammation, cold, fever, snake bite, diarrhoea, dysentery, jaundice and kidney diseases. It is commonly known as Creat or King of bitters in English due to its bitter taste, and Hempedu bumi in Malaysia meaning 'bile of earth' [1]. It is used in Malaysian folk medicine for fever, dysentery, diarrhoea, inflammation and sore throat [2], diabetes and hypertension [3], for treating cardiovascular diseases [4]. It is widely distributed in tropical Asian countries like India, Srilanka, Thailand, Indonesia and Malaysia [5]. It is an

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annual or perennial herb [6] growing up to 1 meter in height with lanceolate leaves and small white to pink, hairy flowers. Various parts of this plant have been reported to possess multiple therapeutic properties including anti-cancer, antidiabetes, anti-inflammatory, anti-malarial, antipyretic, anti-typhoid, antiviral, hepatoprotective, anti-human immunodeficiency virus and immunostimulatory activity [5,7]. Phytochemical studies on the aerial part of the plant showed the presence of a large number of chemical constituents, mainly lactones, diterpenoids, diterpene glycosides, flavonoids, and flavonoid glycosides. Diterpenoids and flavonoids of *A. paniculata* are believed to be responsible for the most biological activities of this plant [8]. Andrographolide a mojor diterpenoid present in the plant is found to be effective in various in vitro conditions of cancer, fever, HIV, inflammation and microbial infections [9]. Priority of A. panicualata is regarded by herbal industries of developing countries such as Malaysia [10], Thailand [11] and is now commercially cultivated to meet the high demand [12]. It is included in priority herbs under the entry point project (EPP) in National Key Economic Areas (NKEA) to develop herbal industry in Malaysia [13]. The commercialization of *A. paniculata* culture is largely hampered due to its limitations of conventional propagation using seeds due to dormancy [14] and poor germination rate and deaths of many young seedlings under natural conditions [15] while vegetative propagation is difficult and considerably slow to meet the commercial requirement of the plants [16].

Biotechnological tools are important for multiplication and conservation of medicinal plants [17]. Plant tissue culture techniques like adventious shooting and somatic embryogenisis are widely used for the production consistent plant material for standardized quality phytopharmaceuticals through mass production. Somatic embryogenesis is the formation of embryos from soamtic cells rateher than fusion of gamates. In this method normal tissues of plant can be turned to embryos under influence of PGRs through a series of morphological and biochemical changes resulting in the formation of a somatic embryo [18]. It serves as a model system in embryological studies [19], as an alternative pathway for mass multiplication of elite cultivars within a short period [20] and also to conserve germplasm and a tool for genetic transformation [21]. Somatic embryogenesis from cotyledons and immature embryos has been widely reported. [22,23,24]. Embryo culture, for many recalcitrant species, is an important milestone for mass micropropagation of plants from a small number of original seeds and a convenient initial source for the establishment of shoot cultures because of their juvenile nature [25]. Synthetic seed or artificial seed contains somatic embryos encapsulated in an artificial seed coat which resembles a normal seed with embryo [26]. The artificial seed coat is made of polymers and even supplemented with nutrients to aid the germination of somatic embryos. Synthetic seeds technology is one of the important applications of somatic embryogenesis in both preservation and practical usage of somatic embryos [27]. In the present study zygotic embryos of *A. paniculata* were used as explants for a rapid propagation through somatic embryogenesis.

2. Materials and Methods

2.1 Sterilization.

Pods were collected from plants grown at agropark in Universiti Malaysia Kelantan and washed thoroughly with running tap water and tween 20 to remove the dust and other surface adhered particles. The pods were then transferred to laminar hood, rinsed with 70% ethanol solution for 30 seconds for thorough cleaning of the pods and ethanol was decanted followed by rinsing with distilled water for 1 minute and water was decanted. 10% sodium hypochlorite and a drop of Tween 20 were used for the second sterilization of the pods for 5 to 10 minutes and rinsed thrice with distilled water for a period of 1 minute.

2.2 Culture of explants.

After sterilization, the pods were placed on an empty sterilized petri dish, opened along the ridges and the seeds were taken out by using the scalpel. Under a dissection microscope, the seed was cut into half and the embryo was removed from the seed. The embryo was then cultured on Murashige and Skoog [28] medium complemented with 30.0 g/L sucrose, 8.0 g/L bacto agar and supplemented with different auxins like NAA, IBA and 2,4-D either alone (1 mg/L, 1.5 mg/L,

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2 mg/L) or in combination of cytokininlike BAP, Kinetin (auxin 1 mg/l + 0.5 mg/L cytokinin, auxin 1.5 mg/l + 0.75 mg/L cytokinin, auxin 2 mg/l + 1.0 mg/L cytokinin) and cytokinin alone TDZ (0.01 mg/L, 0.025 mg/L, 0.05 mg/L, 0.075 mg/L, 0.1, 0.25 mg/L, 0.5 mg/L and 1.0 mg/L).

Table 1: Effect of different PGRs on Callus formation and somatic embryogenesis

PGR mg/L	% callus response	Type of callus	% embryogenic callus	No of somatic embryos per sample
NAA 1	$89.49^{bc} \pm 12.07$	Rhizogenic, friable	-	-
NAA 1.5	$76.43^{d} \pm 0.98$	Rhizogenic, friable	-	-
NAA 2	$98.84^{a} \pm 1.12^{a}$	Rhizogenic, friable	-	-
NAA 1 +Kinetin 0.5	$61.43^{e} \pm 1.43$	Rhizogenic, friable	-	-
NAA 1.5 + Kinetin 0.75	$83.52^{cd} \pm 2.16$	Rhizogenic, friable and embryogenic	20.19 ± 1.41	9.8± 0.6
NAA 2+ Kinetin 1	$43.33^{f} \pm 4.39$	Rhizogenic, friable	-	-
NAA 1 + BAP 0.5	$88.39^{bc} \pm 5.28$	Hard and friable	-	-
NAA 1.5 + BAP 0.75	$95.89^{ab} \pm 3.74$	Hard and friable	-	-
NAA 2 + BAP 1	$93.38^{ab} \pm 2.93$	Hard and friable	-	-
2,4 D 1	$90.00^{abc} \pm 5.29$	Soft and creamy	-	-
2,4 D 1.5	$95.26^{ab} \pm 4.56$	Soft and creamy	-	-
2,4 D 2	$96.67^{ab} \pm 4.16$	Soft and creamy	-	-
2,4 D 1 + BAP 0.5	$94.17^{ab} \pm 2.60$	Soft and creamy	-	-
2,4 D 1.5 + BA 0.75	$92.08^{abc} \pm 4.02$	Soft and creamy	-	-
2,4 D 2 + BAP 1	$98.75^{a} \pm 1.25$	Soft and creamy	-	-
2,4 D 1 + Kinetin 0.5	$89.58^{bc} \pm 5.20$	Soft and creamy	-	-
2,4 D 1.5 + Kinetin 0.75	$95.00^{ab} \pm 1.25$	Soft and creamy	-	-
2,4 D 2 + Kinetin 1	$91.67^{abc} \pm 5.17$	Soft and creamy	-	-
IBA 1	None	None	-	-
IBA 1.5	None	None	-	-
IBA 2	None	None	-	-
IBA 1 + BAP 0.5	None	None	-	-
IBA 1.5 + BAP 0.75	None	None	-	-
IBA 2 + BAP1.0	None	None	-	-
IBA 1 + Kinetin 0.5	None	None	-	-
IBA 1.5 + Kinetin 0.75	None	None	-	-
IBA 2 + Kinetin 1.0	None	None	-	-
TDZ 0.01	None	None	-	-
TDZ 0.025	None	None	-	-
TDZ 0.05	None	None	-	-
TDZ 0.075	None	None	-	-
TDZ 0.1	None	None	-	-
TDZ 0.25	None	None	-	-
TDZ 0.5	None	None	-	-
TDZ 0.75	None	None	-	-
TDZ 1.0	None	None	-	-

2.3 Synthetic seed preparation.

Somatic embryos which are in cotyledonary stage are sepearted from emryogenic callus and transferred to sodium alginate solution 1, 2 and 3 % w/v for synthesis of synthetic seeds. The embryos covered with sodium alginate is solidified in 100 mM calcium chloride for 10 minutes and stored.

3. Results and Discussion

Indirect somatic embryogenesis has been observed in the current study and the embryos germinated into plantlets. Of all the PGRs used somatic embryos were formed only in NAA 1.5 mg/L + Kinetin 0.75 mg at an average mean of 9.8± 0.6 per explant and percentage embryogenesis was 20.19 ± 1.41% (Table 1). While organogenic callus is observed in both NAA alone and NAA with Kinetin and white hard friable callus was formed in NAA supplemented along with BAP. In a research by Martin [29] 2.69 µM NAA + Kinetin 1.16 µM was used for formation of somatic embryos from calluses induced on media fortified with 5.37-10.74 µM NAA and Kinetin 2.32 or 4.65 µM and usage of NAA with Kinetin for somatic embryogenesis have been reported earlier in other plants [29,30]. 2,4-D is the most commonly used auxin in induction of somatic embryos and in a previous report on A. panicualta [16] it induced but in the present study when 2, 4-D either used alone (1, 1.5, 2 mg/L) or in combination of

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cytokinin viz BAP and Kinetin (2,4-D 1 mg/l + 0.5 mg/L cytokinin, 2,4-D 1.5 mg/l + 0.75 mg/L cytokinin, 2,4-D 2 mg/l + 1.0 mg/L cytokinin) there is no formation of somatic embryos, the callus was creamy which turn into brown around 40 days and when subcultured further in same media still there was no formation of embryos or conversion into organogenic callus which was different from other research in which leaf callus formed somatic embryos on 2, 4 D [16]. The same type of results were observed in Pisum sativum [31] and Arachis hypogeae [32], NAA was more efficient than 2,4-D for inducing primary somatic embryogenesis. On the other hand there was no formation of embryos or callus in media supplemented with IBA alone, IBA+BAP, IBA+Kin and TDZ, while they resulted in germination of the zygotic embryos.

Plant propagation in vitro via somatic embryogenesis or organogenesis is a complicated process requiring the proper execution of several steps, which are affected by choice of the explants such as part, age, physiological state, culture conditions and environment [21,33]. The use of immature embryos to induce somatic embryos in other dicotyledonous plants proves the efficacy of this explant as a good target tissue for embryogenic material [34]. According to Neumann [35] the explant of choice is the zygotic embryo followed by hypocotyls, petiole, leaf lamina and finally root. It is not surprising that the embryogenic potential is highest in zygotic embryos since zygotic embryo cells are with many of the genes required for the induction process as they are already expressed in them.

Somatic embryogenesis is typically induced within callus by exogenous auxin as they participate in the regulation of developmental switches [36,37]. Auxins and cytokinins are used for in vitro media most frequently, probably because they synergistically participate in cell division and cell differentiation respectively that induce an embryogenic pathway [33,37,38]. In most instances in which cytokinines induced somatic embryogenisis, they were added to the culture medium together with auxins [33]. However, in some cases, the addition of cytokinins as the sole source of PGR is sufficient to generate somatic embryos [39,40].

Synthetic seeds prepared from sodiuam alginate 3% is firm and round in shape compared to 1 and 2 %, and were cultured in MS medium supplemented with BAP 0.5 mg/L, NAA 0.05 mg/L and GA3 2 mg/L.

4. Conclusion

Embryos can produce somatic embryogenesis in a faster way than other explants due to the embryogenic potential of the cells. In the present study lower concentrations of NAA and Kinetin produced somatic embryos may be because of their organogenic abilty which suppressed embryo formation at higher concentration. This method is rapid and efficient to produce somatic embryos from *Andrographis panicualata* which in turn could be converted into synthetic seeds, these synthetic seeds can be used for cryopreservation and general usage as planting material.

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