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Induction and proliferation of adventitious roots from *Aloe vera* leaf tissues for *in vitro* production of aloe-emodin

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Abstract

We have inspected aloe-emodin and aloin compounds in different tissues of *Aloe vera* which were grown in Aloe farm for three years. Surprisingly, aloe emodin contents were much richer in the roots (574.8 ± 92.4 µg/g) than in leaves (5.52 ± 0.32 µg/g) that encouraged us to establish adventitious root culture system of the plant. The optimal condition for induction and proliferation of adventitious roots using young *Aloe vera* leaves was established by treatments of variety of conditional media and auxin supplements. Adventitious root induction was suitable by enrichment of 0.5 mg/L 1-naphthalene acetic acid (NAA) and 0.2 mg/L 6-benzylaminopurine (BA) in Murashige & Skoog (MS) medium. However root proliferation was hindered by accumulation of phenolic compounds in the media that was overcome by pre-washing of the adventitious roots with more than 4 g/L of polyvinylphyrollidine (PVP) analogs increasing the survival rate (up to 60 %). Inspection of aloe-emodin contents in various adventitious roots grown different basal medium revealed that aloe-emodin accumulation is much higher on B5 medium (133.08 ± 0.12 µg/g) than on MS medium (3.56 ± 0.26 µg/g).

Keywords : Adventitious roots; Aloe-emodin; *Aloe vera*; BA; NAA; PVP.
Abbreviations : BA - 6-Benzyl aminopurine; B5 media - Gamborg B5 medium; IAA - Indole-3 acetic acid; IBA - Indole-3-butyric acid); MS media - Murashige & Skoog medium; NAA - 1-naphthalene acetic acid; HPLC – High performance liquid chromatography; PVP - Polyvinylphyrollidine; SH media - Schenk & Hildebrandt medium; 2,4-D - 2,4-dichlorophenoxyacetic acid.

Introduction

Aloe belongs to the family Liliaceae and is a perennial tropical plant originated from Africa. The genus Aloe includes approximately 500 species. Among them, only five species, *Aloe vera*, *Aloe arborescens*, *Aloe perryi*, *Aloe ferox* and *Aloe saponaria* are mainly used for medicinal purposes. Among them, *Aloe vera* is the most popular for commercial and therapeutic purposes (Park and Lee, 2006). The representative compound in *Aloe vera* is an anthraquinone, aloe-emodin, synthesized via the polyketide pathway (Han et al., 2001). Aloe-emodin has been shown to exhibit efficacy for anti-inflammatory and genetic toxicity properties (Park and Lee, 2006). The leaves contain more than 98.5-99.5% water that indicated trace amounts of secondary metabolites residing in the leaf tissues, even after three years of cultivation for commercial harvest (Femenia et al., 1999). Moreover, the medicinal composition and activity can be easily decreased by different environmental and physiological conditions, especially those that hinder the stable productions of certain compounds (Beppu et al., 2004). Plant cell cultures are an alternative way to obtain useful secondary metabolites and have been studied in many plant species. The differentiated organ culture, especially adventitious root cultures, have been applied in many medicinal plants due to its rapid growth and stable mass production ability of secondary metabolites (Murthy et al., 2008). Adventitious root systems have been established for production of anthraquinone in *Morinda citrifolia* (Baque et al., 2010) and for an anthraquinone compound, lucidin-3-*O*-primeveroside, in *R. tinctorum* (Sato et al., 1997). Some study was conducted for development of *Aloe vera* micropropagation system (Hashem and Kaviani, 2010) but there was no previous report for adventitious root induction and proliferation. In this study, we have found *Aloe vera* roots grown for three years in aloe farm contain large amounts of aloe-emodin that encouraged us to estabilish adventitious root culture system.Here we report an optimized condition for adventitious root induction and proliferation using young *Aloe vera* leaf tissues. Moreover, we have identified aloe-emodin contents are remarkably increased in adventitious roots grown on B5 culture media than others that will promote in vitro production of the compound.

Results and discussions

Quantification of aloe-emodin and aloin in different organs of normally grown Aloe vera plant and regenerated adventitious roots

We compared the aloe-emodin and aloin contents in different

Table 1. Contents of aloin and aloe – emodin in adventitious root, leaves and roots of *Aloe vera.*

Sample	Aloin (µg/g)	Aloe-emodin (µg/g)
Adventitious root	0	3.6 ± 0.3^{b}
Root	3.9 ± 0.2^{a}	574.8 ± 92.4^{a}
Leaf	1499.1 ± 904.0^{b}	5.5 ± 0.3^{b}

Adventitious root were grown for six weeks in MS media including NAA 0.5 mg/L + BAP 0.2 mg/L, and roots and leaves were collected from plants grown for three years in an aloe farm field.

Table 2. Effects of NAA concentration on adventitious root induction from leaf explants of *Aloe vera*.

NAA concentration (mg/L)	Induction rate (%)	Number of roots per explant	Root length (cm)
0.1	23	6.5 ± 0.3^{b}	2.7 ± 0.2^{b}
0.5	52	10.1 ± 0.2^{a}	4.1 ± 0.3^{a}
1.0	27	5.1 ± 0.4^{c}	1.9 ± 0.1^{c}

Significantly different at a 5% level

Table 3. Effects of basal media on aloe–emodin content in aloe adventitious roots.

Media	Dry weight/explants (g)	Aloe-emodin (µg/g)
MS	0.1	3.6 ± 0.3^{c}
1/2MS	0.09	4.3 ± 0.5^{b}
SH	0.05	5.7 ± 0.1^{b}
2MS	0.06	6.4 ± 0.03^{b}
B5	0.07	133.1 ± 0.1^{a}

Each medium has the same hormone combination, NAA 0.5 mg/L + BAP 0.2 mg/L.

organs of *Aloe vera* including leaves and roots of *Aloe vera* which were normally grown in aloe farm for three years and adventitious roots which were obtained from initial adventitious root induction experiments using various hormone combinationss. They were analyzed in order to determine the potential of *in vitro* mass production of the compounds using adventitious root culture system. Interestingly, aloe-emodin and aloin were found not only in leaves, but also in roots of three-year-old *Aloe vera* (Table 1). The aloin content was 1,499.1 ± 904.0 µg/g in leaves. A small amount of aloin (3.9 ± 0.2 µg/g) was detected in roots of three-year-old aloe and nothing was detected in the adventitious roots. Meanwhile, the content of aloe-emodin was 574.8 ± 92.4 µg/g in roots, remarkably higher than that of leaves (5.5 ± 0.3 µg/g). Furthermore, 3.6 ± 0.3 µg/g of aloe emodin was detected in adventitious roots grown for seven weeks in basal media including NAA 0.5 mg/L + BAP 0.2 mg/L. The concentration was similar to that of the three-year-old leaves, indicating that adventitious root culture has strong potential to produce aloe secondary metabolites via *in vitro* culture of adventitious roots.

Induction of Aloe vera adventitious roots

In order to determine the optimal conditions of *Aloe vera* for adventitious root induction, we tested various kinds and concentrations of auxin supplements (2,4-D, NAA, IAA and IBA) on MS media. NAA was the only phytohormone to induce adventitious roots from leaf explants after three weeks of culture. No adventitious roots were initiated in auxin-free media. Old leaves and shoots greater than 10 cm in size did not induce adventitious roots under any conditions. Supplementation with IBA and IAA showed no or very little adventitious root induction, respectively. An easily friable callus was induced by supplementation of 2,4-D in basal MS media. Significant effects of NAA concentration were detected for adventitious root induction. Supplement of lower concentrations, such as 0.1, 0.5 and 1.0 mg/L was good for adventitious root induction, while increasing to higher concentrations, 2.0, 4.0 and 6.0 mg/L resulted in induction of a friable callus. Collectively, NAA 0.5 mg/L was the most effective for bringing about improvements in induction rate, number of adventitious roots per explant, and root length during six weeks of culture (Table 2). Although adventitious roots were induced by adding NAA 1 mg/L, the roots were callus-like, short, and fragile (Fig. 1B). Dry weight was greater at NAA 1 mg/L (0.13 ± 0.03 g) in MS media than at NAA 0.5 mg/L (0.058 ± 0.01 g). Many studies have indicated that NAA activates cell division and root induction. NAA treatments induced adventitious roots and activated elongation of lateral roots in *Lycopersicon esculentum* (Taylor et al., 1998). A high concentration of NAA induced callus-like, short adventitious roots in *Karwinskia humboldtiana* (Kollarova et al., 2004), as in our results for supplementation with more than 1 mg/L NAA, indicating that an overdose of NAA may reduce organogenesis. Meanwhile 5 mg/L IBA showed positive effects on adventitious root accretion compared to those of 7 and 9 mg/L NAA in *Morinda citrifolia* (Baque et al., 2010).

Proliferation of adventitious roots

Even though NAA 0.5 mg/L supplementation was the best condition to induce adventitious roots, biomass increase was not best under this condition. Therefore, we determined the optimal condition for adventitious root growth. Adventitious roots induced for three weeks on MS media culture with NAA 0.5 mg/L supplementation were transferred to media with additional supplementation of BAP 0.1, 0.2 or 0.3 mg/L (Fig. 2). Dry weight increased for the first five weeks of culture and decreased thereafter in most treatments. Dry weight was increased by increasing the NAA concentration up to 1.0 mg/L. However, callus-like adventitious roots were induced at 1.0 mg/L of NAA, and their dry weights were rapidly decreased after five weeks. On the contrary, dry weight increased gradually for the first six weeks of supplementation of BAP 0.2 mg/L in addition to NAA 0.5 mg/L (0.106 ± 0.001 g) (Fig. 2).

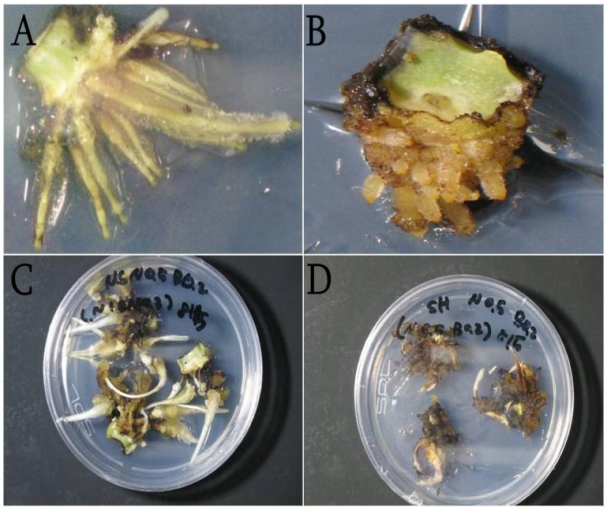

Fig 1. Different features of *Aloe vera* adventitious roots based on different NAA concentrations (A, B) and different media (C, D). A and B, adventitous roots cultured for three weeks on MS media supplemented with different concentrations of NAA. NAA 0.5 mg/L (A) and NAA 1.0 mg/L (B) supplementation resulted in normal and callus-like adventitous roots, respectively. C and D, adventitous roots cultured for six weeks on different media, MS (C) and SH media (D) with the same hormone NAA 0.5 mg/L + BA 0.2 mg/L.

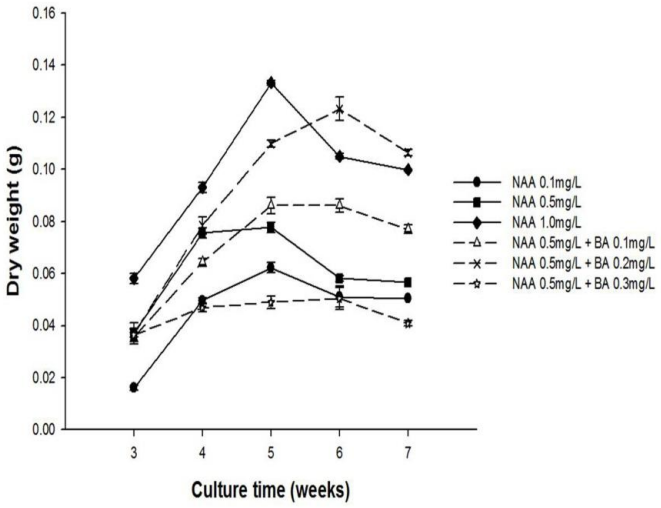

Fig 2. Effects of different hormone supplements on dry weight increase in *Aloe vera* adventitious root culture during the first seven weeks. Six hormone combinations, NAA 0.1, 0.5, 1 mg /L, NAA 0.5 mg/L + BA 0.1 mg/L, NAA 0.5 mg/L + BA 0.2 mg/L, NAA 0.5 mg/L+ BA 0.3 mg /L, were supplemented on MS media.

We also compared biomass increases based on different kinds of media, 2x MS, MS, half strength-MS (1/2 MS), SH, and B5, with the same supplementation of NAA 0.5 mg/L + BAP 0.2 mg/L. The best adventitious root growth was observed in MS media (0.123 ± 0.002 g) (Fig. 1C, Fig. 3), which was twice as great as that in SH media (0.061 ± 0.002 g) (Fig. 1D, Fig. 3). Ammonium nitrogen is quickly consumed in plant tissues and is directly metabolized. However, excess ammonium is very toxic, inhibiting cell metabolism. Low ammonium nitrogen vs. nitrate nitrogen ratio was shown to be important for the induction of adventitious roots in ginseng root culture (Han et al., 2006). Similarly, *Echinaceae angustifolia* root growth rate was best in 1/2 MS media with a 5:25 ratio of ammonium nitrogen vs. nitrate nitrogen (Wu et al., 2006). However, aloe adventitious roots showed a higher growth rate in MS media, rather than in other media with lower ammonium nitrogen concentrations, implying that ammonium nitrogen is not critically inhibitive for aloe adventitious root culture, even though more a precise ammonium nitrogen vs. nitrate nitrogen ratio should be determined to conclude this assumption.

Inhibition of browning

When adventitious roots were separated from the primary induction media and cultured individually, the roots severely browned and died. This was the critical problem for tissue culture of aloe species. Therefore, it is necessary to prevent explants browning to achieve successful mass production of *Aloe vera* adventitious roots. Roy et al. (1991) reported that supplementation of PVP in the media inhibited browning in aloe tissue culture. However, we did not obtain any significant improvements with these additions. A significant delay in browning was observed after pre-washing the adventitious roots in PVP analogues prior to the transfer to the second growth media. Adventitious roots pre-washed with various PVP analogous survived, regardless of PVP analog type, washing time or pH value. However, PVP concentration substantially affected survival rate, indicating that viability increased by pre-washing tissue with a solution containing more than PVP 4.0 g/L of analogs (Fig 4). Pre-washing mango explants via agitation in PVP solution was very efficient not only for inhibition of browning, but also for prevention of leakage of phenolic compound in excised explants (Krishna et al., 2008). Pre-washing, including with glutathione, also reduced total phenolic compounds and PAL activity in pistachio, resulting in significant growth increases (Tabiyeh et al., 2005). We assumed that tissue pre-washing with PVP removed phenolic compounds secreted from excised explant surfaces to aid in the adaptation of the explants in the new media for second growth. Therefore, the aloe roots transferred after pre-washing in PVP solution survived, and lateral roots were induced in MS media without growth hormone. Meanwhile, adventitious roots were not induced when PVP was directly supplemented on root induction media.. Many phenolic compounds released from excised surfaces might induce cell necrosis. Pre-washing of the excised explants with PVP removes these compounds and results in reduced cell damage, thus promoting root initiation. However, addition of PVP in initial root induction media resulted in absorption of phenolic compounds that might negatively affect the initial induction of adventitious roots in *Aloe vera*.

Comparison of aloe-emodin contents in adventitious roots grown different growth media

We compared aloe-emodin contents in adventitious roots grown on different basal media (Table 3). No significant difference in aloe-emodin content was detected between two different adventitious roots when they were grown on the same MS media with different hormone combinations, such as NAA 0.5 mg/L + BAP 0.1 mg/L and NAA 0.5 mg/L + BAP 0.3 mg/L. Therefore, we compared five basal media with the same hormone combinations to clarify the effects of increasing aloe-emodin content. The results showed that aloe-emodin content was dramatically increased (133.08 ± 0.12 μg/g) in the adven-

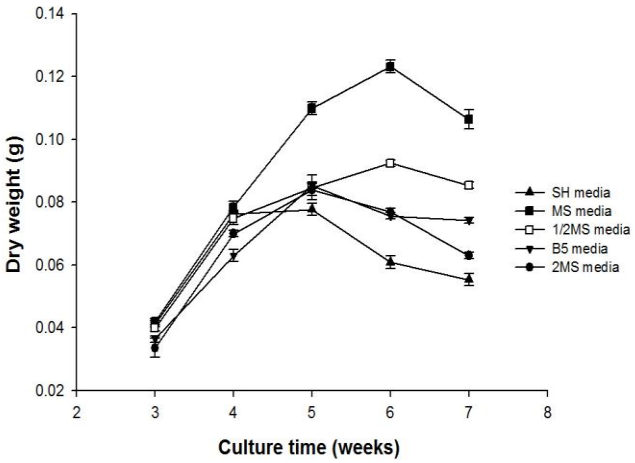

Fig 3. Effects of basal media on dry weight increase in *Aloe vera* adventitious root culture during the first seven weeks. Three different media, 2xMS, MS, and 1/2 MS, were compared in a supplement mixture of NAA 0.5 mg/L + BA 0.2 mg/L.

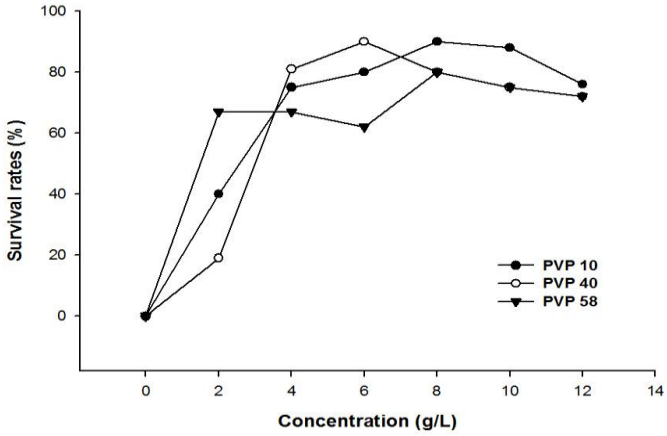

Fig 4. Effects of various concentrations of PVP analogs during pre-washing of the adventitious roots. Same stage adventitious roots were separated from the primary adventitious root induction media and were washed via agitation in the PVP solution in order to inhibit browning and were sub-cultured on MS media without growth hormone.

titious roots grown in B5 media to 20~40 fold higher than that those in the other media (Table 3). Increased ammonium nitrogen concentration not only inhibited biomass increase but also accumulated useful phytochemicals, even though preferences for nitrate nitrogen and ammonium nitrogen components vary by species (Kronzucker et al., 1999). Adventitious root growth and phenolic compound accumulation in *Echinacea angustifolia* was maximized in modified MS media, which had 5 and 25 mM of ammonium nitrogen and nitrate nitrogen contents, respectively (Wu et al., 2006). Adjustment to a 1 : 5 (w/w) ammonium nitrogen : nitrate nitrogen ratio in MS media was shown to be optimal for artemisinin accumulation in *Artemisia annua* L. hairy root culture, but ammonium itself inhibited biomass and artemisinin production (Wang and Tan, 2002). Our experiments showed slight difference for *Aloe vera* adventitious root growth dependent on basal media. However, a critical increase in aloe-emodin was detected in the adventitious root grown in B5 medium which had the lowest ammonium concentration. Hairy root growth of *Lobelia inflate* occurred in the order of MS > 1/2 MS > B5 media, but NN medium (Nitsch and Nitsch, 1967) showed more lobeline synthesis in fresh weight (Yonemitsu et al., 1990). Even though B5 showed the best aloe-emodin production in our experiment, further conditions should be inspected. Providing two-step cultures that differ for adventitious root growth and useful chemical production may be considered because the observed growth was the best in ammonium-rich MS medium, but aloe-emodin content was the best in ammonium-free B5 medium. Further experiments should be performed to determine the possibility of mass production of adventitious roots in suspension culture. Further experiments for adjustment of nitrogen sources and optimal secondary metabolite production upon treatment of various elicitors will contribute to establish an efficient root culture system for *in vitro* mass production of useful metabolites using *Aloe vera* adventitious roots. Collectively, our data have shown an optimal adventitious root culture system and the potential for mass production of the key functional compound, aloe-emodin, using the system. The next step will be achievement of mass production of adventitious root cell lines and optimal accumulation and purification of the compound to produce functional and commercial products using the system.

Materials and methods

Explants preparation and culture conditions

All the aloe plants were provided from Kim Jeong Moon Aloe Co. Ltd (Jeju, Korea). Young leaves of approximately 10-cm-tall shoots emerged from suckers of 3 to 5-year-old *Aloe vera* plants were used as explants for adventitious root induction. The leaves were surface-sterilized with 70 % ethanol for 30 s, immersed in 4 % sodium hypochlorite for 5 min and rinsed three times with sterile distilled water. Sterilized explants were excised into 1 x 2 cm sections and were cultured on plastic Petri dishes (12 x 1.5 cm) containing 25 ml basal medium with 0.7 % agar supplemented with 3 % (w/v) sucrose. The pH of the medium was adjusted to 5.8 before autoclaving at 121 ℃ for 15 min. Cultures were maintained at 28 ± 1 ℃ in the dark.

Adventitious roots induction

The leaf explants were cultured on full-strength MS medium supplemented with various concentrations (0, 0.1, 0.5, 1, 2, 4, 6 mg/L) of NAA, IAA, IBA, and 2,4-D in order to determine the optimal auxin and their concentration. Different concentrations (0.1, 0.2, 0.3 mg/L) of BAP supplements were compared in combination with 0.5 mg/L NAA. Different kinds of media, such as SH (Schenk and Hildebrandt, 1972), B5 (Gamborg *et al.*, 1968), 2 MS, MS, and 1/2 MS, were compared to identify the best conditions for growth and aloe-emodin accumulation in adventitious roots. Growth patterns of each treatment were measured every week for six or seven weeks of culture duration. All experiments included eight replicates per treatment, and the experiment was repeated ten times.

Inhibition of browning

Browning due to accumulation of phenolic compounds in the media is the biggest problem for the growth of aloe adventitious roots. To determine the effect of PVP pre-treatment on browning inhibition and promotion of survival rate, explants were prewashed with various concentrations (0, 2, 4, 6, 8, 10, 12 g/L) of different PVP analogues, PVP 10

(Duchefa, Netherlands), PVP 40 (Sigma, United states), PVP 58 (Acros, United states), washing times (0, 10, 20, 40, 60, 120 min) and pH conditions (2.0, 2.5, 3.0, 3.5, 4.0) before being cultured in MS media without hormone. All experiments were conducted with four replicates per treatment, and experiments were repeated ten times.

Quantification of aloe-emodin and aloin

We quantified aloe-emodin and aloin based on the method of Park et al. (1998). Leaves and roots of three-year-old *Aloe vera* plants and seven-week-old adventitious roots induced from *Aloe vera* leaf were analyzed using high performance liquid chromatography. Freeze-dried material (0.5 g) was prepared in ethanol (10 ml) for 1 hr at 50 ℃ using a JAC ultrasonic 2010 sonicator (Jinwoo Engineering, Korea) and suspensions were centrifuged at 2330 g for 15 min. The supernatants were filtered through 0.45 μm (Whatman) membrane filters. Hewlett Packard Series 1100 HPLC and a Shiseido capcell pak C18 column (4.6 x 250 mm, 5 μm) were used for analysis. The separation was conducted using 25~30% methanol for 5 min, 30~35 % methanol for 10 min, 35~70 % methanol for 35 min and 70% methanol for 10 min at 0.7 ml/min. The peaks were monitored using a diode array detector (DAD) at 293 nm at room temperature. The data represent the means of three replicates.

Statistic analysis

We compared media conditions with four to ten replications and quantify compounds in different organs with three replications. Analysis of variance (ANOVA) was used to ascertain significant differences between treatments using the Statistical Analysis System (SAS 9.1). Multiple comparisons of treatment means were conducted using Duncan's test or the Least Significant Distribution (LSD) test at $P < 0.05$.

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