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Enhanced Shoot Organogenesis in *Aloe saponaria* Following Treatment with Ethylene Inhibitors and Polyamines

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Plants belonging to the genus *Aloe* are wide spread in Africa and are important in the pharmaceutical industry. Here, we investigated the role of ethylene inhibitors and polyamines in the enhancement of shoot regeneration from meristem explants of *Aloe saponaria*. A rapid, highly effective shoot regeneration procedure was designed using meristem explants. First, we found that Murashige and Skoog (MS) with 2mg/L6-benzyl amino purine (BAP) was the best for shoot initiation. Shoot regeneration was assessed using different concentrations of $AgNO_3$, aminoethoxyvinylglycine (AVG), and $CoCl_2$ (1, 5, 10, and 20mg/L). While $AgNO_3$ induced shoots (3.5/explant) and increased shoot length by 2.5cm at concentrations of 10mg/L, an additional increase in the concentration of $AgNO_3$ decreased shoot production and shoot length. We also analyzed the effects of three different polyamines (putrescine,spermidine, and spermine) at various concentrations (10, 30, 70, and 100mg/L), in addition to MS with 2mg/L BAP, on shoot regeneration and length. Among these compounds, putrescine best augmented shoot regeneration and length, with the largest increase in the number of shoots (3.7/explant) and a moderate augmentation in shoot length 2.5cm at 70mg/L using MS with 2mg/L BAP, followed by spermidine and spermine. This study revealed that the addition of ethylene inhibitors and polyamines could improve shoot regeneration and length in *Aloe* species. The procedures established in the present study could be used to produce a larger number of shoots, as well as enhance plant growth over a short period, and could be used as an important tool in future gene transfer studies.

Key words: *Aloe saponaria*, Meristem explants, Micropropagation, Ethylene inhibitors, Polyamines.

The genus *Aloe*, which belongs to the Xanthorrhoeaceae family, includes over 500 known species, of which, *Aloe saponaria*, is one of the most important species in Africa. This speciesis commonly known as soap aloe or zebra aloe and is a salt tolerant plant widely occurring in South Africa, Zimbabwe, and America. Recently, *Aloe* species have been extensively studied and are also being used in the pharmaceutical industry for their medicinal value[1] and mainly in the manufacture of soaps for foam production. Additionally, *A. saponaria* has a less bitter taste compared to other *Aloe* species and has recently become the most favored plant in California for its ornamental value. However, *A. saponaria* is difficult to identify as it hybridizes easily with other *Aloe* species; its leaves are red or green and are characterized by an "H" shaped spot in the leaves. Furthermore, *A. saponaria* populations are sensitive to changes in temperature and water.

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Currently, the study of human diseases has been increasing worldwide, and new medicines for controlling disease have been developed. *Aloe* species have medicinal properties; however, owing to their low population, the global demand for these species is high. The gel extracted from *A. saponaria* leaves contains polysaccharides, proteins, fats, aloin, and resins[2]. Polysaccharides of *A. saponaria* have immuno modulatory activities, as has been reported in both *in-vivo* and *in-vitro* experiments[3]. Furthermore, previous studies on *A.saponaria* demonstrate that this plant can have haemagglutinating properties and can prevent the multiplication of tumor cells while also improving the normal human cell growth cell. In particular, the compound mannan from *A.saponaria* has been shown to inhibit tumor cell activation and proliferation[4]. Aloe species have both wound healing and anti-inflammatory properties[5-7], as well as antinociceptive and anti-inflammatory properties[8].

In recent years, the demand for *Aloe* species has increased due to its medicinal importance. It is very difficult to induce plant proliferation due to its low growth rate *in-vivo* conditions. Thus, tissue culture plays an important role in plant regeneration and micropropagation. The *in-vitro* plant micropropagation and regeneration of aloe species has been previously reported[9-11], with the *in vitro* regeneration of *A. saponaria* using meristem explants previously explored[12]. Ethylene is a volatile compound produced in excessby plants and affects plant growth. Ethylene inhibitors have been used *in vitro* in order to improve cell growth and regeneration rates. In particular, ethylene inhibitors increase shoot regeneration and cell growth in *Aloe arborescens*[13], as well as in the hypocotyl explants of the Chinese radish[14]. Both aminoethoxyvinyl glycine (AVG) and AgNo3 enhanced shoot regeneration in mustard[15]. In Chinese cabbage, polyamines improved the regeneration frequency[16] and the use of these compounds to induce embryogenesis was investigated[17].

The currently available protocol for the *in-vitro* regeneration of *A. saponaria* using meristem explants is inefficient and leads to a very low frequency of shoot regeneration, with no improvement in cell growth. In this study, we report the development of an improved method for enhanced shoot regeneration and shoot length from meristem explants of *A. saponaria* using different concentrations of ethylene inhibitors and polyamines.

MATERIALS AND METHODS

Shoot organogenesis from meristem explants

One-year-old A. saponaria *meristem explants were collected from the green house of Chungnam National University, washed in running tap water for 30 min, surface-sterilized with 70% (v/v) ethanol for 30 s and 1% (v/v) sodium hypochlorite solution for 10 min, then rinsed three times in sterilized water to remove the sodium hypochlorite solution. The sterilized meristem explants were cut aseptically at the ends, into four sections approximately 0.7 cm in size. Meristem explants were placed on a Petri dish (100 × 25 mm) with approximately 25 mL of culture medium. Seven explants were cultured in each Petri dish. The basal medium consisted of MS[18] medium and was solidified with 0.7% (w/v) Phytagar. The pH was adjusted to 5.8 before adding Phytagar. The media were sterilized by autoclaving at 1.1 kg* cm^{-2} *(121 °C) for 20 min. For shoot regeneration from meristem explants, the MS medium consisted of 0, 0.1, 0.5, 1, 2, or 4 mg/L kinetin and BAP. In order to improve shoot regeneration, the medium was optimized by testing the effect of various concentrations of anti-ethylene agents (0, 1, 5, 10 or 20 mg/L of* $AgNO_3$*, AVG, and* $CoCl_2$*) on shoot formation and growth. Enhancement of shoot organogenesis was tested at different concentrations of polyamines (10, 30, 70, and 100 mg/L of putrescine, spermidine, and spermine). Inoculated cultures were maintained at 25 °C in a growth chamber with a 16-h photoperiod under standard cool white fluorescent tubes (35 mmol* s^{-1} m^{-2}*) for 6 weeks. The shoots were transferred to a new MS medium every 15 days.*

Rooting of regenerated shoots

Regenerated shoots (approximately 1cm long) were placed in MS medium, which was solidified with 3g/L Phytagar and dispensed at 30 mL per Magenta box, with four shoots cultured in each box. Regenerated shoots were incubated at 25°C in a growth chamber with a 16h photoperiod under standard cool white fluorescent tubes (35

mmol s^{-1} m^{-2}) for 5 weeks. After six weeks, the rooted plants were washed with sterile water to remove Phytagar, transferred to pots containing autoclaved vermiculite, and covered with polyethylene bags for one week to maintain conditions with high humidity. The plants were then transferred to soil and maintained in a growth chamber with a 16-h photoperiod, and a day/night temperature of 20/18 °C for 2 weeks. These plants were then transferred to the greenhouse.

RESULTS AND DISCUSSION

Effect of different ethylene inhibitors on shoot regeneration and shoot length

Overall, the addition of ethylene inhibitors improved shoot regeneration as well as shoot length. In this study, the effect of three different ethylene inhibitors and different concentrations was assessed. *A. saponaria* cultured on MS medium with 2mg/L BAP produced significant changes in shoot regeneration. After 15 days, the inoculated culture began to regenerate from the meristem explants. A significant effect was observed with the addition of 10 mg/L of $AgNO_3$ and AVG; however, further increase in the concentration of $AgNO_3$ and AVG decreased shoot regeneration rates and shoot length. The maximum shoot regeneration and shoot length was obtained at 1/2 MS with 2mg/L of BAP and 10mg/L of $AgNO_3$, with slightly inferior results observed with the addition of AVG, and no improvement under treatments with different concentrations of $CoCl_2$ showed in Table 1.

Effect of different polyamines on shoot regeneration and shoot length

To evaluate the effect of polyamines at different concentrations on shoot regeneration of *A. saponaria*, meristem explants were grown for 4 weeks. The addition of polyamines increased shoot number and shoot length, with a significant improvement using putrescine at 70mg/L in shoot regeneration and shoot length and no further improvement observed with a further increase in concentration. The highest shoot number and shoot length was induced by putrescine at 70mg/L, followed by spermidine and spermine at the same concentration (Table 2).

Plant tissue culture is the main tool for micropropagation and regeneration of various plants. In order to establish a plant regeneration system, we investigated the effects of different ethylene inhibitors and various concentrations of anti-ethylene agents ($AgNO_3$, AVG, and $CoCl_2$) as well as polyamines (putrescine, spermidine, and spermine) on the efficiency of shoot organogenesis in ***A.saponaria.*** Previously, a protocol was established for shoot organogenesis

Table 1. Effect of different ethylene inhibitors on shoot formation and growth of *Aloe saponaria* on MS media supplemented with BAP 2 mg/l

Ethylene inhibitors(mg/L)		No. of shoots /explant	Shoot length(cm)
Control	0	2.3 ± 0.2	1.5 ± 0.1
$AgNO_3$	1	2.5 ± 0.3	1.6 ± 0.1
	5	3.1 ± 0.3	1.9 ± 0.2
	10	3.5 ± 0.3	2.3 ± 0.2
	20	2.1 ± 0.2	1.3 ± 0.1
AVG	1	2.4 ± 0.2	1.5 ± 0.2
	5	2.9 ± 0.3	1.7 ± 0.2
	10	3.3 ± 0.4	2.1 ± 0.2
	20	2.2 ± 0.3	1.4 ± 0.1
$CoCl_2$	1	2.3 ± 0.2	1.4 ± 0.1
	5	2.6 ± 0.3	1.6 ± 0.2
	10	2.0 ± 0.2	1.4 ± 0.1
	20	1.7 ± 0.2	1.1 ± 0.1

The values are the means ± SD

Table 2. Effect of different polyamines on shoot formation and growth of *Aloe saponaria* on MS media supplemented with BAP 2 mg/L

Ethylene inhibitors(mg/L)		No. of shoots /explant	Shoot length(cm)
Control	0	2.3 ± 0.2	1.5 ± 0.1
Putrescine	10	2.6 ± 0.2	1.7 ± 0.2
	30	3.2 ± 0.3	2.0 ± 0.2
	70	3.7 ± 0.4	2.5 ± 0.3
	100	3.5 ± 0.3	2.1 ± 0.2
Spermidine	10	2.3 ± 0.2	1.4 ± 0.1
	30	2.5 ± 0.3	1.6 ± 0.2
	70	2.6 ± 0.3	1.8 ± 0.2
	100	2.1 ± 0.2	1.5 ± 0.2
Spermine	10	2.2 ± 0.2	1.5 ± 0.2
	30	2.3 ± 0.2	1.4 ± 0.2
	70	2.5 ± 0.3	1.5 ± 0.2
	100	1.9 ± 0.2	1.3 ± 0.1

The values are the means ± SD

and plant regeneration from meristem **explants** of ***A.saponaria***[12], in which the regeneration of aloe species using MS medium with auxins and cytokinins at different concentration[19] was assessed. More recent studies reported that ethylene induces regeneration and shoot elongation in selected plants[20]. Ethylene inhibitors have been shown to improve regeneration and shoot length[21] in *A. arborescens* and $AgNO_3$ enhances micropropagation and regeneration frequency[22]. Since in many cases plant regeneration is affected by the increased production of ethylene[23], we investigated the use of ethylene inhibitors in the regeneration medium and found that the accumulation of ethylene reduced the regeneration frequency. We established a regeneration method for *A. saponaria* using different ethylene inhibitors at various concentrations. We found that $AgNO_3$ at 10mg/L induced the highest number of shoots (3.5 per explant [Table 1]). AVG and CoCl2 produced fewer shoots. In addition, we studied the ability of polyamines to regenerate *A. saponaria*. It has been previously reported that polyamines improve shoot regeneration in *Cucumis sativus* and *Withania somnifera*[24,25]. Furthermore[26] investigated the addition of spermidine, which significantly improved shoot proliferation in apricot leaves, and [27]demonstrated that putrecine induced root development in *Hedera helix*. In this study, we used three different polyamines (putrescine, spermidine, and spermine), and found that putrecine producesthe greatest number of shoots. Both spermidine and spermine were less efficient compared to putrecine. We also devised a highly efficient system involving the use of meristem explants, ethylene inhibitors, and polyamines for the *in-vitro* micropropagation of *A. saponaria*. To our knowledge, this is the first study that uses polyamines for the regeneration of *Aloe* species.

CONCLUSION

In conclusion, plant regeneration protocols are essential for inducing genetic transformation and accelerating plant growth. Currently, shoot organogenesis is extensively used for *in-vitro* plant regeneration and transformation. The protocol established in the present study is an effective method for regenerating *A. saponaria* as well as producing additional plants within a short period of time. Remarkably, the ethylene inhibitors $AgNO_3$ and AVG promoted the frequency of shoot organogenesis in this species. In addition, polyamines enhanced the shoot number and length. Together, our findings potentially form the basis for the improvement of genetic tools for *Aloe* species.

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