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Effect of abiotic elicitors on tissue culture of *Aloe vera*

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Abstract

Aloe vera L. belong to the Aloaceae family and actually is a commercial and medicinal important plant because of the secondary metabolites. Anthraquinones are well- known and widely used in medicine and food chemistry. It has dermatological usage with antimalarial, antifungal and antibacterial properties. Aloin is the most important secondary metabolite in *Aloe vera*, not only due to the medicinal and cosmetic properties but also due to their physiological role. Plant cell cultures have been used industrially for the synthesis of secondary metabolites. In this study we have investigated the effects of different abiotic elicitors including Nano-Ag, Nano-Tio2, NH_4NO_3 and sucrose on cell suspension culture of *Aloe vera*. The induced callus by elicitors were collected in five period of time and have been analyzed by HPLC (High-performance liquid chromatography). The results showed that the highest amount of aloin obtained with NH_4NO_3 at 48hours after treatment, and it was 127% higher than control. Aloin content was enhanced with Nano elicitors at 48 hours after treatment and decreased gradually after that. Moreover, aloin was increased gradually up to168 hours after sucrose treatment. These experiments showed that in vitro culture of *Aloe vera* can be induced to produce more secondary metabolites.

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Introduction

Most of bioactive compounds in medicinal plants belong to secondary metabolites which are usually less abundant than primary metabolites in the plants (Chong *et al* , 2005; Smetanska 2008). Cell suspension cultures are the best system of cultivation for producing secondary metabolites because fast growth rates can be achieved. Several biotechnological advances have been developed in tissue culture that improve secondary metabolites production such as optimization of cultural conditions, selection of high-producing strains of lines, precursor feeding, metabolic engineering, transformed root culture and elicitation (Sarin, 2005). Elicitors are chemicals or biofactors from various sources that can induce physiological change of the target living organism. In a broad sense, "elicitors", for a plant refer to chemicals from various sources that can trigger physiological and morphological responses and phytoalexin accumulation (Zhao *et al*, 2005). Biotic and abiotic elicitors are used to stimulate secondary metabolite product concentrations and increased culture volume (Ramachandra and Ravishankar, 2002) .The different types of abiotic elicitors are shown in table 1.

The field of nanotechnology is one of the most active areas of research in modern material science (Jain *et al,* 2009). Nanoparticles are materials that are small enough to fall within the nanometric range with at least one of their dimension beings less than a few hundred nanometers. This reduction in size brings about significant changes in their physical properties with respect to those observed in bulk materials (Gonzalez-Melendi *et al,* 2008). Silver nanoparticles (Ag-NPs) are among the most commercialized nanoparticle due to their antimicrobial potential and are wildly used in medicine, physics, material science and chemistry (Asharani *et al,* 2009). It is generally recognized that silver nanoparticles may attach to the cell wall, thus disturbing cell wall permeability and cellular respiration. It is also possible to penetrate the nanoparticles within cells and interfere with the phosphorus and sulfur of DNA and proteins (Elumalai *et al,* 2010). Ag-NPs have several antimicrobial functions to control various phytopathogen. Few studies have reported both positive and negative effect of nanoparticles on higher plant. Studies on the seed germination and root growth of *Zucchini* plants in hydroponic solution amended with Ag NPs showed no negative effects whereas a decreased in plant biomass and transcription was observed on prolonging their growth in presence of Ag-NPs (Stampoulis *et al,* 2009) .The cytotoxic and genotoxic impact of Ag-NPs were studied using root tips of onion. It was investigated that Ag-NPs impaired the stages of cell division and caused cell disintegration (Kumari *et al,* 2009). Nanoscale Tio_2 and sio_2-enhanced nitrate reductase activity in Soybean, and apparently hastened its germination and growth (Lu *et al,* 2002). Nano-Tio_2 improved light absorbance and promoted the activity of Rubisco activase thus accelerated Spinach growth. Nano-Tio_2 improved plant growth by enhanced nitrogen metabolism that promotes the absorption of nitrate in spinach and accelerating conversion of inorganic nitrogen into organic nitrogen, thereby increasing the fresh weights and dry weights (Nair *et al,* 2010).

Aloe vera L is an important medicinal plant from *Aloaceae* family with African origin. Among 300 species, *Aloe vera* is considered as an important medicinal plant in many countries (Hasanuzzaman *et al*, 2008; Reynolds, 2004). The two major liquid sources of *A. vera* are yellow latex and clear gel, which is obtained from the large parenchymatic cells of the leaf (Ni *et al*, 2004). The gel of *A.vera* possesses various biological and physiological activities in cosmetology and medicine, healing ability of skin burns and cutaneous injuries, prophylactic effect against radiation leucopenia, antiulcer, inhibitory action against some bacteria and fungi, inflammation-inhibiting effect, inhibition of the prostaglandin synthesis by anthraquinone-type compounds, and inhibition of the AIDS virus by acemannan (Hamman, 2008; Hernandenez *et al,* 2002; Ramachandra and Srinivasa, 2008). The main constituents of the latex are anthraquinones. Aloin an

active compound obtained from the Aloe vera species is already confirmed to exhibit on anti-inflammatory, anticancer effect.

Aloin, also known as Barbaloin is a bitter yellow-brown colored compound noted in the exudate of at least 68 Aloe species at levels from 0.1 to 6.6% of leaf dry weight (making between 3% and 35% of the total exudates) and in another 17 species at indeterminate level(Arun P *et al*, 2012). Interaction between nitrogen (N) and Benzyladenine (BA) resulted in increased aloin concentration and chlorophyll content in *Aloe vera* that the highest levels of aloin concentration and chlorophyll content were obtained in N1500+ BA1000 ppm and N1500 mg per pot absolutely, respectively (Hazrati *et al*, 2012).The objective of this study was to determine the effects of different abiotic elicitors on the production of aloin.

Materials and methods

Collection of plants and preparation of calli

Fresh *Aloe vera* plants were collected from Medicinal Plant Research Institute. They were cut into 10±1 mm segments, and used as explants from base of leaves. The explants were disinfected with 1% benomyl for 20 min, and washed with sterile distilled water and then in 70% alcohol for 30 s and 5% NaOCl for 15 minutes followed by three sequential rinses for 5 minutes in sterile distilled water. The sterilized pieces of leaves were subjected into 1/2 *Murashige and Skoog medium* (MS medium) with 1 mg/L NAA for callus initiation (Fig 1). All of the cultures were transferred to fresh media at 3-week intervals and after 3 months, the best calli were selected and transferred to suspension.

¼ Nitrate and ½ MS basal media without agar were used for suspension culture. The calli were transferred into 150 conical flasks containing 50 mg of liquid media. The cultures were incubated on a rotary shaker operated at 100 rpm and 25° C (Fig 2).

Elicitation

To increase aloin production, 412.5 mg/ml NH_4NO_3, 100 g/L sucrose,120 mg/L Nano Tio_2 and 0.625 mg/ml Nano Ag were added in suspension cultures and collected after 6, 24, 48, 7, and168 hours.

Aloin concentration assay

The calli were collected and freeze dried at -10° C for 24 h. The aloin content was quantified by HPLC technique (Model, 2487 Waters, USA) using a Bondapack™ C 18µ(4.6 X 250 mm, dp 10µm) reversed-phase column (Phenomenex, 4.6 mm i.d. x 250 mm length, 5 µm). The mobile phase (flow 0.7 $ml.min^{-1}$) was composed of methanol (HPLC grade, Merck) and water and UVS detectors. An aloin stock solution (5000 ppm) was made up in a 1:1 of methanol/water. The solvents were selected 500, 100, 80, 50 and 25 ppm. The accuracy of the calibration curves for aloin was tested using reference samples with known concentration of the compounds.

Aloe powder (20 mg) was dissolved in 2 ml methanol and water (1:1) passed through a C18 cartridge to selectively extract only the phenol fraction. Injection volume is true 20 µl. The chromatography was obtained by using HPLC equipped with a C18 column (4.6×250 mm, dp 10 µm). A diode array detector with two channels was used (channel A set at 275 nm, channel B set at 365 nm).

Statistical analysis

Data were statistically analyzed by one way analysis of variance. For comparing significant differences of set of means, Duncan test was applied at $P<0.05$ using SPSS version 21 software.

Results and discussion

Aloe Vera L has a different secondary metabolites and the most important of them is Aloin. Aloin is the active components that has anti-ulcer, inhibiting action against some bacteria and fungi-inflammation, healing ability of skin burns and cutaneous injuries properties. The aim of the present research was to evaluate the effects of elicitors on production of Aloin in *Aloe vera* cell suspension culture.

The results showed that, treatment of aloe

suspension cell cultures with Nano-Ag caused the aloin content increased up to 43.7% in 48 hours after treatment then decreased gradually and reached to the control level. The highest amount of aloin obtained by Nano- Tio_2 treatment at 48 hours and after that, this level was reduced at 168 hours (Fig 3, Fig 4).

Table 1. Classification of abiotic elicitors.

Elicitors	Plant cell culture	Elicited product	Refrence
Methyl Jasmonate	Taxus sp	Paclitaxel, Taxanes Diterpenes	Patel and Krishnamurthy R.2013
Ag-NPs	Salvia miltirorrhiza	Tanshinone production	Zhao JL *et al*,2010 Zhang CH *et al*,2004
	Taxus chinensis	Paclitaxl production	Choi HK *et al*,2001
	Brugmansia candida hairy root	Tropane alkaloids	Pitta-Alvarez SL *et al*,2000
	Saussurea medusa	Flavonoid Jaceosidin Hispidulin production	Zhao D-X *et al*,2005
Amonium nitrate	Vitis vinifera	Phenolic content (Resveratrol)	Lee-Sae *et al*,2011
Copper Sulphate	Digitalis Lanata	Cardiac glycoside Flavonoids	Bota C Deliu C,2011
Copper and Cadmium Salt	Datura Stramonium	Sesquiterpenoid	Furze JM *et al*,1991
Salicylic Acid	DaucusCarota	Chitinase	Patel H and Krishnamurthy R.2013
Jasmonic Acid+ Salicylic Acid+Ethephone	Vitis vinifera	Phenolic content	Riedel H *et al*,2012

Nano-Ag is a new class of material with remarkably different physiological and biological characteristics such antimicrobial, antifungal and antiviral activities. This nanoparticle accelerates the aloin rate in 48 hours after elicitation but after that, this level was diminished gradually and reached the control level. Ag-NP elicitation was related to the released dissolved Ag^+ and nanoparticle. It might be related to feedback of Aloin on the expression of genes, and high level of Aloin is a cause of decreased DNA expression. That result was confirmed in a study on production of taxol on *Taxus baccata* by Asghari *et al* in 2012. In that study, Nano-Ag and *salicylic acid* had negative effect on production of taxol in *Taxus baccata*. But atremisinin content in the cultures was increased by stimulation of Nano-Ag (Zhang *et al,* 2013).

Another useful nano elicitor is Nano-Tio_2 which could promote significantly photosynthesis and greatly improve growth of Spinach by the change of nitrogen metabolism (Yang *et al,* 2006). That nano elicitor could increase the aloin content in 48 hours after elicitation but reduced to lower level, 8.8%, than control. The decline might be related to the toxic effect of Nano-Tio_2 in the medium culture or impact of that nanoparticle on gene expression. However, both of nano elicitors enhanced the aloin content 48 hours after treatment but thereafter decreased gradually.

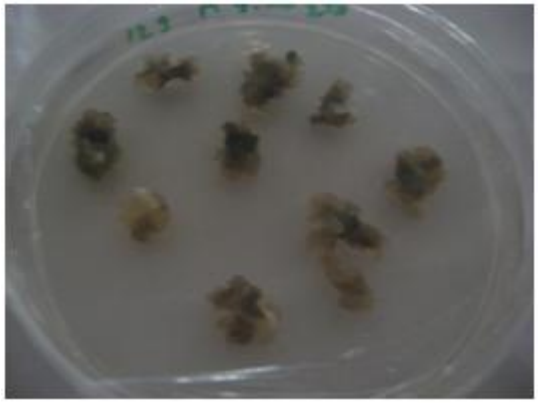

Fig. 1. The callus in 1/2MS medium with 1 mg/L NAA.

HPLC determination showed that elicitation with NH_4NO_3 could accelerate the amount of aloin 127% at 48 hours after elicitation and then it was reduced gradually and reached 112% at 168 hours. This rise might be due to the influence of NH_4NO_3 on the expression of genes which are involved in the production of aloin pathway.The similar result was obtained in other study that NH_4NO_3 increased *Artemisian* amount on the suspension culture in *Artemisia annua* (Bladi and Dixit, 2008) and also *Lee* and his colleague in 2011 demonstrated that 5000 mg/L NH_4NO_3 augmented biomass and accumulation of secondary metabolites 21 days after treatment in *Vitis vinifera* (Fig 5).

Fig. 2. ¼ Nitrates and ½ MS basal medium without agar was used for suspension culture.

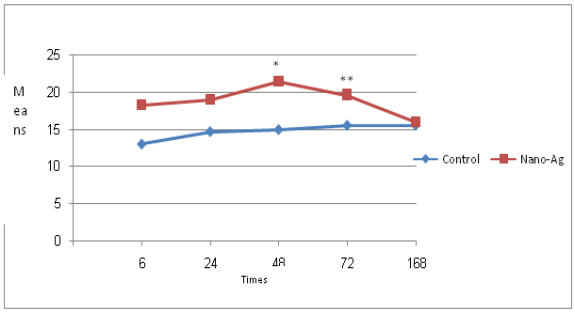

Fig. 3. Effect of Nano-Ag on the callus in cell suspension culture.

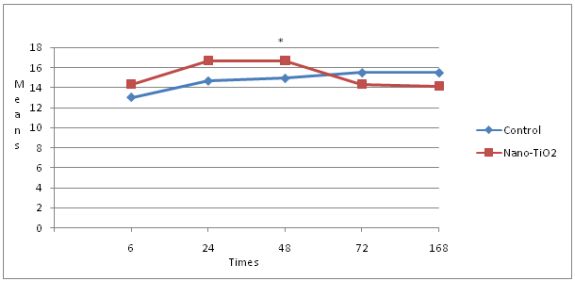

Fig. 4. Effect of Nano-Tio$_2$ on the callus in cell suspension culture.

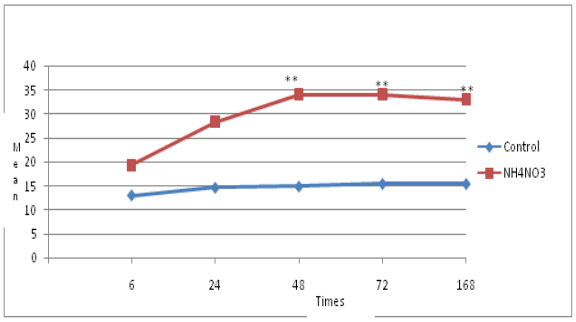

Fig. 5. Effect of NH_4NO_3 on the callus in cell suspension culture.

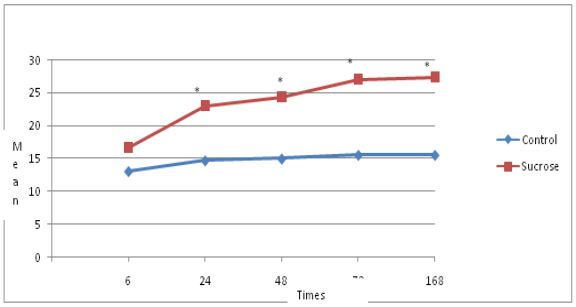

Fig. 6. Effect of Sucrose on the callus in cell suspension culture.

Sucrose is one of the abiotic elicitors in this study. The aloin content began to increase rapidly during 168 hours. The highest content was 76.3% at 168 hours after elicitor usage. It might be related to the role of sucrose as a precursor in the production of aloin pathway (Mulabagal and Tsay, 2004). In another study, 2.5% and 7% sucrose caused an increase in the rosmarinic acid up to 0.8 and 3.3 g/L (Missava *et al*, 1998) (Fig 6).

Conclusion

From the above-mentioned results, it could be concluded that elicitation is effective method in the synthesis of secondary metabolites. Therefore, in order to strengthen the secondary metabolites in *Aloe vera*, usage of abiotic elicitors can be suggested.

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