ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 9, Suppl. 1, 2016

Online - 2455-3891
Print - 0974-2441

Research Article

MICROPROPAGATION AND ELICITATION STUDIES IN *ALOE VERA*

NISHITA SHUKLA, NAGASHREE N RAO, ASHWANI SHARMA*

Department of Biotechnology, R.V. College of Engineering, Bengaluru, Karnataka, India. Email: ashwanisharma@rvce.edu.in

Received: 23 July 2015, Revised and Accepted: 28 May 2016

ABSTRACT

Objective: Micropropagation and elicitation studies of Aloe vera

Methods: Standardisation of the regeneration protocol was done by using Murashige and Skoog (MS) media in combination with various plant growth regulators and elicitation studies were carried out by means of abiotic elicitors (salicylic acid, fructose and sodium chloride).

Results: Direct regeneration through shoot tip gave the best response (0.2 mg/l Indole Butyric Acid (IBA) + 0.2mg/l 6-Benzylaminopurine (BAP)). Salicylic acid proved to be an effective abiotic elicitor, for suspension cultures of *in vitro* grown callus and also *in vivo*. It increased the content of polysaccharides, up to 7 times and phenolic compounds to almost 10 folds compared to untreated control kept under similar conditions.

Conclusion: This study will make a path for the genetic transformation and metabolite enhancement along with their regulation, which can prove to be an asset towards pharmaceutical industry.

Keywords: *Aloe vera*, Succulent plant, Micropropagation, Elicitation, Callus, Explant.

INTRODUCTION

Aloe vera, a plant with many benefits, can rightly be called as a "Medicinal Miracle." It is widely used for various purposes, ranging from health to cosmetic applications. It contains numerous bioactive components that prove to be beneficial for humans in a variety of ways. This particular species has been frequently quoted for its use in herbal medicine since the first century [1]. With increasing knowledge and technology, it has become one of the most widely exploited plant species, in terms of natural remedies. The extract derived from *A. vera* leaves is a common constituent of cosmetics and other alternative medicines and is used by industries which market such drugs for their rejuvenating, healing, or soothing properties.

A. vera or *Aloe barbadensis* Miller is a plant of family Liliaceae [2] and also called as "Burn plant." It is a xerophyte which can be easily grown even in lands with low levels of rainfall. It is mainly propagated using suckers (or) offshoots. It takes around a year to produce a commercial crop [3]. The leaves of this plant are known to comprise more than 200 bioactive constituents such as saponins, anthraquinones, mucopolysaccharides, steroids, vitamins, and glucomannans [4-6]. The diverse pharmacological and therapeutic effects of *A. vera* is hypothesized to be associated with the heterogeneous composition of the *A. vera* pulp [7]. The two major chemical constituents of the plant are anthraquinones present in the exudate which contributes to most of the medical benefits and polysaccharides that are responsible for numerous properties imparted by the gel.

Intense research is on to identify and isolate various metabolites from the gel, as well as to identify new properties, which can create new medical breakthroughs such as inhibiting cell proliferation in cancer cells and treatment of atherosclerosis. In the food industry, it has been extensively used as a source of functional foods and as an ingredient in other food products, for the production of gel-containing health drinks and beverages. In the cosmetic and toiletry industry, it has been used as base material for the production of creams, lotions, soaps, shampoos, facial cleansers, and other products. In the pharmaceutical industry, it has been used for the manufacture of topical products such as ointments and gel preparations, as well as in the production of tablets and capsules [8,9]. Recent development in pharmaceutical applications suggests that both the *A. vera* gel and whole leaf extracts have the ability to improve the bioavailability of co-administered vitamins and other drugs in human subjects [10,11]. All these wide ranges of applications have led to an increasing demand of the plant, which can be met by micropropagation, and the efficiency of the plant as a medicinal agent can be enhanced using elicitation. Hence, the present study was conducted to achieve regeneration, rapid multiplication, and shoot proliferation with enhances metabolite production.

In vitro regeneration and propagation of this plant has been carried out by various groups of researchers [12-20] using different explants and different media compositions. However, meager reports are available for callus culture of *Aloe* species because of the pertinent problem of primary cultures establishment due to the high phenolic secretion by the wounded edges of the explant. Although, there are some reports on callus induction and morphogenesis in this species [15,21-23], which are not reproducible due to environmental intricacies. Through this study, a standardized optimization of the media design was attained that controlled the phenolic secretion effectively and also led to successful and reproducible shoot proliferation or organogenesis, either directly through the explant or through callus induction. Various compositions of auxins and cytokinins in Murashige and Skoog (MS) media were tested, along with different types and age of explants.

Along with regeneration, short-term *in vitro* and *in vivo* elicitation studies were also carried out in *A. vera* to enhance its important active metabolites targeting mainly polysaccharides and phenols/anthraquinones. Elicitation mechanism in *A. vera* is not well studied. Unfortunately, not much work has been done regarding elicitation in *Aloe* sp., especially *in vivo*/in field to evaluate short-term elicitation effects using abiotic elicitors. Long-term effect of different factors such as water, sunlight, and nitrogen has been evaluated over long period [24-27], but lack of short-term studies persists. The results of this study can prove to be highly beneficial for improving commercial value of this plant and its efficacy as a medicinal/cosmetic agent. This is the first report, in which direct and indirect regeneration along with elicitation of the same explants has been exhibited.

METHODS

Micropropagation

Disease-free, healthy plant material was procured from Dhanvantari Nursery, Bengaluru University. Initial analysis of the parent plant was done from which explant was to be derived for content of polysaccharides and phenolic content using simple colorimetric tests such as anthrone test for polysaccharides [28] and phenol estimation by Folin-Ciocalteu's (F-C) reagent [29]. The explants were trimmed to suitable size of 2-3 × 10^{-2} m and then washed thoroughly in running tap water for 30 minutes and Tween 20 (Merck, India) for 10 minutes followed by thorough rinsing to remove any traces of detergent for 10 minutes. 1% w/v solution of Bavistin (BASF India, Limited) was used as a fungicide treatment for 15-20 minutes. After proper rinsing explants were taken inside the laminar air flow for surface sterilization with freshly prepared 0.1% w/v aqueous solution of mercuric chloride for 1-2 minutes, with vigorous shaking. Thereafter, they were washed using autoclaved distilled water 2-3 times. The explants were further trimmed to 0.5-1 × 10^{-2} m segments and aseptically transferred to tissue culture bottles containing solidified MS media supplemented with different plant growth regulators such as auxins, cytokinins, and other adjuvants (Plant growth regulator [PGRs] - using 1 × 10^{-3} g/ml stock solutions of the PGR) to test which composition works the best. Growth conditions were maintained at 20-25°C with 8/16 photoperiod.

To check media browning due to phenolics, the explants were frequently subcultured to fresh media so that the growth was not hampered. Subculturing was done for tissues that exhibited proliferation and growth after 28-30 days of initial inoculation. However, once the growth rate accelerated, the explants were transferred to fresh media every 10-15 days.

Elicitation studies

In vitro elicitation studies

For *in vitro* studies, callus was initiated on the margins of the leaf sections of the plant by cultivating them on solidified MS media supplemented with 1 × 10^{-3} g/L of 2,4-dicholoroacetic acid and 0.2 × 10^{-3} g/L of 1-naphthaleneacetic acid. Adenine sulfate (20 × 10^{-3} g/L) was used as an antioxidant to overcome the persistent problem of browning of the explant and the media. The initial proliferation was visible after 2 weeks of inoculation (Fig. 1a) and was quite prominent by the end of 4^{th} week (28 days) (Fig. 1b).

The callus culture was aseptically transferred to autoclaved liquid MS media with the same additives as used for callus induction. Before transferring, some of the leaf sections were subjected to analysis of polysaccharide and phenolic compounds. These data were recorded as "0^{th} hr" results, which were later used to compare the results of elicitation. These leaf sections were transferred to 18 different conical flasks having 40 ml liquid MS media with 3-4 leaf sections (~2 g of leaf sections). Each treatment including control was carried out in triplicates. Salicylic acid (SA) was used as abiotic elicitor in two different concentrations of 3 × 10^{-6} mol and 5 × 10^{-6} mol. All the flasks were kept in shaker incubator and subjected to 96 hrs and 144 hrs of agitation at 25°C and 130 rpm.

After completion of the due time, the flasks were removed from the incubator, and the leaf sections were separated from media and homogenized with little quantity of acetone, in a mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 10 minutes. The supernatant collected was left to evaporate residual acetone. The concentrated supernatant was then used for analysis. The samples were directly analyzed for content of polysaccharides using anthrone reagent, and the methanol extract of the supernatant was used as sample for analysis of phenolic compounds using F-C reagent. 0.2 ml of the sample (supernatant) was diluted to 1 ml with distilled water for analyzing.

In vivo elicitation studies

In vivo elicitation studies were carried out on wild-type potted plants, procured from Dhanvantari Nursery, Bangalore University. The elicitors used were: 100 × 10^{-3} mol sodium chloride (NaCl), 100 × 10^{-3} mol fructose, and 100 × 10^{-3} mol SA for a period of 7-day and 15-day. Plants of almost similar growth (Fig. 2) were taken for analysis and grouped into four categories of three plants each, which are – Group 1 (untreated - watered with 90 ml plain water), Group 2 (watered with 90 ml of 100 × 10^{-3} mol NaCl), Group 3 (watered with 90 ml of 100 × 10^{-3} mol fructose), and Group 4 (watered with 90 ml of 100 × 10^{-3} mol SA).

Before the commencement of the study, "Day 0" analysis was done to check the content of polysaccharides and phenolic compounds in the starting plant material and compare with the results post-study. For this, one matured leaf was excised from each plant and used for analysis. The supernatant obtained after centrifugation of homogenized leaf was used for analysis. The plants were kept in proper sunlight and aeration in open ground. These plants were watered thereafter, with water every alternate day. On the 7^{th} day and 15^{th} day of treatment, leaves were excised from each plant and prepared for analysis. Post-analysis, the data were recorded and graphically interpreted to report the effect of the study on the useful metabolites of *A. vera*.

RESULTS

Micropropagation

For direct regeneration, the different types and age of explants used. These were: Young shoot tip, rhizome apex of a matured plant, and nodal region of underground rhizome.

Direct regeneration was obtained using MS media supplemented with 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA). For shoot initiation and proliferation, 0.2 × 10^{-3} g/L of each BAP and IBA was used. Later for further multiplication and elongation of shoots

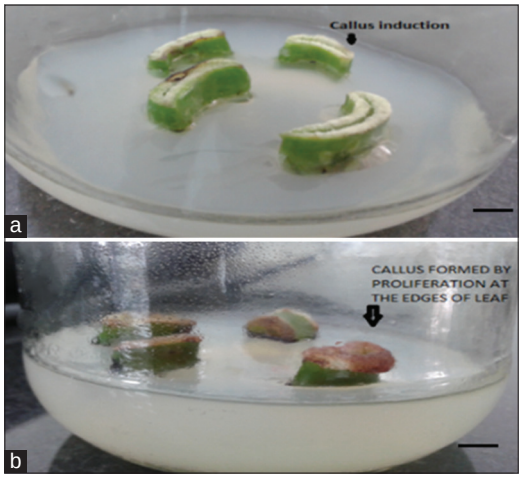

Fig. 1: (a) Callus induction after 2 weeks, (b) callus growth after 4 weeks (Bar represent 1 cm)

Fig. 2: Plants kept for *in vivo* elicitation study

and rooting, the young plantlets were transferred to media containing IBA (0.2×10^{-3} g/L) and BAP (2.0×10^{-3} g/L). Each of the explants gave results for shoot proliferation at the initial stage, but the growth was fastest in case of young shoot tip where the shoots of about $1\text{-}1.5 \times 10^{-2}$ m were formed in about 15 days' time. The rhizome apex and nodal region of rhizome showed early signs of growth, but shooting occurred only after 30-40 days. The number of young shoots arising from rhizome apex was maximum (~8-10), but the shoot growth was very poor (Fig. 3).

The number of shoots arising from nodal region was minimum (~3-4) with a good growth, but rooting of such plantlets was difficult due to a higher risk of contamination while separating the plantlets from the base (Fig. 4).

In all manners, young shoot tip was found to be the best explant choice for direct regeneration, with faster growth, healthy plantlets, more number of secondary plantlets arising from the regenerated plant, moderate number of shoots (5-6), good capability of rooting and acclimatization, and lesser chances of contamination (Fig. 5).

Repeated subculturing to fresh media was done after every 10-15 days. After 2 months, rooting of plantlets was observed. Once, the roots reached to 3-4 in number and $3\text{-}4 \times 10^{-2}$ m in length (after 20 days of root initiation), the plantlets could be successfully acclimatized (Fig. 6).

During initial 1.5 months, the best control of phenolic secretions was given by citric acid (0.2 g/L). For later stages, i.e., shoot elongation and rooting the media was supplemented with 2 g/L of activated charcoal (powdered) to control the phenols and enhance growth.

All these results and findings help to standardize the various available protocols for regeneration and come to a single concrete solution for direct regeneration and micropropagation of *A. vera.* Through the above-tested media composition for various stages of growth, many plantlets can be regenerated from a single shoot tip especially that of a young plant in lesser time and space. To compare the regenerated or *in vitro* grown plant with the parent plant from which explant was taken, the plants were observed morphologically and also analyzed for important metabolites. The micro propagated plants were morphologically similar to parent plants, in terms of leaf texture, thickness and also with respect

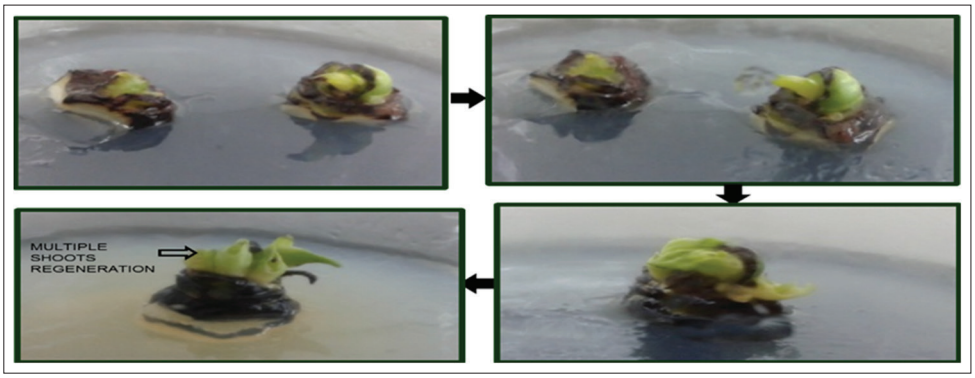

Fig. 3: Multiple shoot regeneration stages from a matured rhizome apex

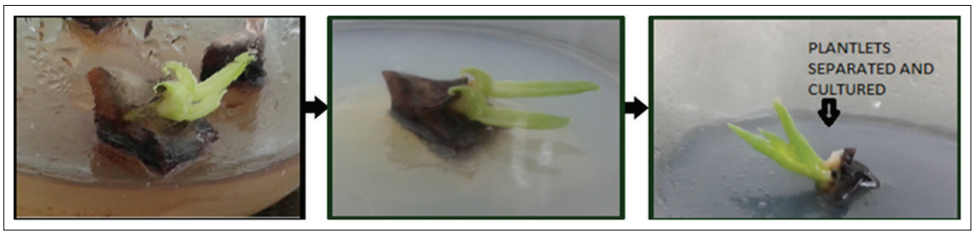

Fig. 4: Shoot development stages from nodal region of underground rhizome

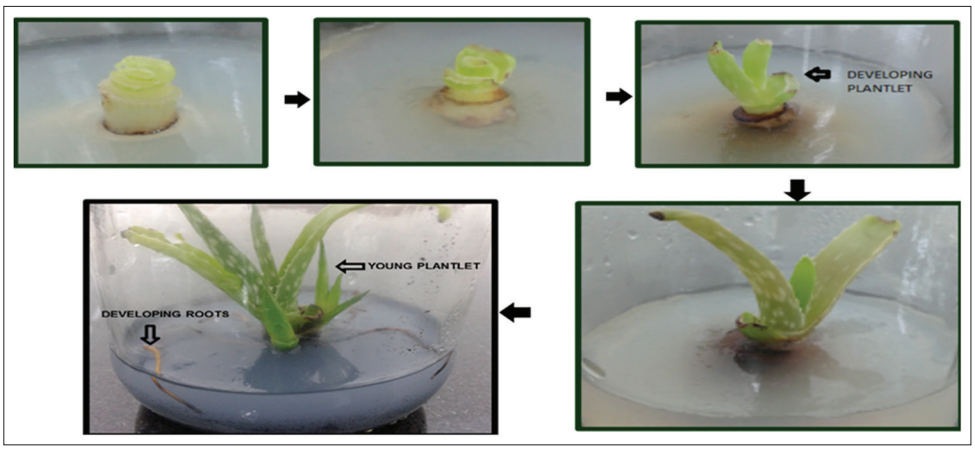

Fig. 5: Stages of regeneration of plantlet from young shoot-tip

to the root length. Test for polysaccharides and phenolic compounds that were conducted to assess any changes in the metabolite content of the regenerated plant gave the results that are expressed in Figs. 7 and 8 using bar graphs.

The results obtained suggest that the regenerated plant is also metabolically similar to the parent plant or rather superior to it in terms of metabolites. The slight increase in metabolite content exhibited by the results may be due to the PGRs added into the culture media, which act as elicitors for these metabolic pathways and have a direct influence on the phytochemical composition of the plants [30].

Fig. 6: Growing acclimatized plant

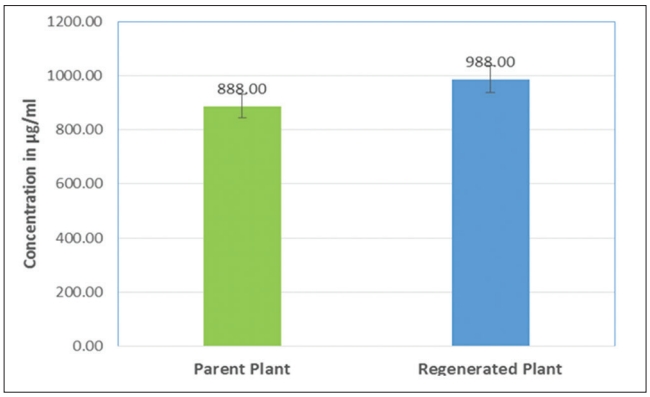

Fig. 7: Polysaccharide content in parent and regenerated plant; p≥0.05

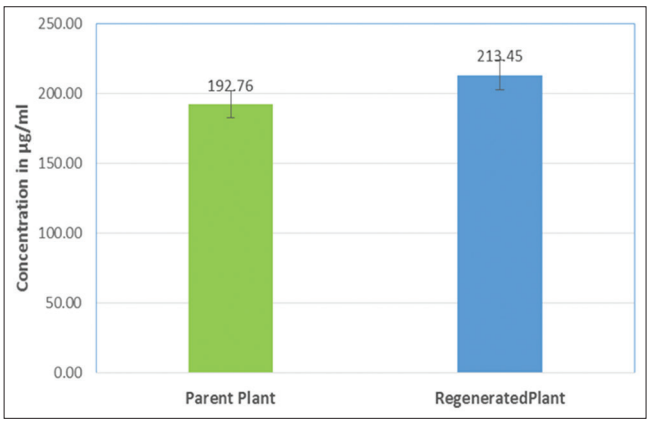

Fig. 8: Phenolic content in parent and regenerated plant; p≥0.05

Elicitation studies

Non-genetic elicitation was carried out using abiotic elicitors for callus cultures - *in vitro* and for naturally grown plants - *in vivo.* The results were obtained in terms of the change in metabolite concentration in treated and untreated plants during the stipulated time-period. The estimation of metabolites – phenolic compounds and polysaccharides in the samples were done using the equations of linearity obtained from the standard graphs.

In vitro elicitation studies

For analyzing the metabolite content (polysaccharides and phenols), samples were prepared from these 28 to 30-day-old callus cultures of treated and untreated groups. The treatment group consisted of 3×10^{-6} mol and 5×10^{-6} mol of SA as abiotic elicitor which was used to study the response after 96th hr and after 144th hr of initiation of the study. Polysaccharide content was tested using anthrone reagent and phenolic compounds by F-C reagent. The results obtained are expressed in terms of the "fold change" in concentration of metabolites from 0th hr to 96th hr, 0th hr to 144th hr, and from 96th hr to 144th hr of the study. Fig. 9 indicates fold change in polysaccharide content in the respective groups, w.r.t control. The results show almost 2-10 folds increase in the content of polysaccharides at the end of 96-144 hrs of study as compared to the content present before treatment. However, the polysaccharides have increased by 5-10 folds in the treated groups, whereas in the untreated group the increase is only 1.5-2 times. Furthermore, the rate of increase (per hour or per day) is more in the group treated with 5×10^{-6} mol of SA but more rapid during the first phase (0-96th hr) than in the second phase of the study (96-144th hr).

Fig. 10 indicates fold change in phenolic content in the respective group w.r.t control.

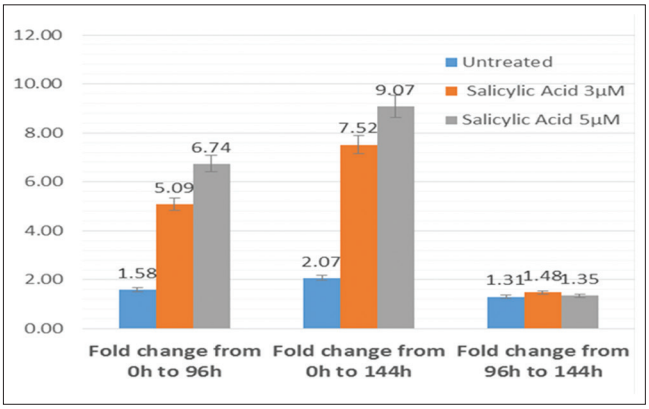

Fig. 9: Fold change of polysaccharide content with respect to 0th h control in untreated and treated groups; p≥0.05

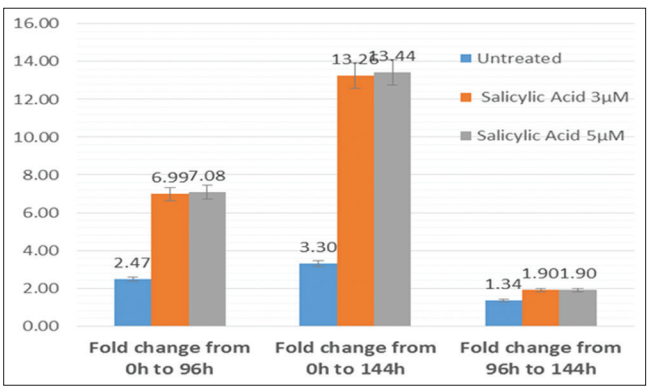

Fig. 10: Fold change of phenolic content with respect to 0th h control in untreated and treated groups; p≥0.05

The fold change in content of phenolic compounds w.r.t. 0th hr control is within 2.5-13 amongst various groups. The fold change is much less in case of untreated group (2.5-3.3 folds) than in treated groups (7-13 folds), even though there is no significant difference in the data obtained for 3×10^{-6} mol and 5×10^{-6} mol of SA treatment. The rate of increase is more up to 96 hrs (0.07 µg/ml/h) in case of treated groups than the rate of increase after 96 hrs of elicitation.

To summarize, from Figs. 9 and 10, it is clearly evident that the fold change in the metabolite content of the callus cultures, is quite significant (almost 5-10 folds for polysaccharides and 7-13 folds for phenolic compounds) when treated with SA. Even the cultures treated with 3×10^{-6} mol, SA show a considerable increase w.r.t 0th hr control when compared to the untreated group. Thus, SA was found to be an effective abiotic elicitor for secondary metabolite synthesis in our study.

Therefore, the efficacy of *A. vera* as a medicinal agent can be highly improved through SA treatment in suspension cultures.

In vivo elicitation studies

The effect of different elicitors (100×10^{-3} mol NaCl, fructose, and SA) was assessed by comparing the polysaccharide (anthrone test) and phenolic content (F-C test) of the treated and untreated group of plants with the initial content present in the plants before commencement of the study at "Day 0." Since the plants were naturally growing plants, the initial content of polysaccharides cannot be the same for all the plants. Although care was taken to select plants of similar growth, it was practically impossible to have all the plants of the same metabolic profile. Hence, the grouping was done in a way that plants belonging to one group were quite alike in terms of growth, but slightly different from plants of other groups. To address this, discrepancy every group was compared against its own control, i.e., "Day 0" data obtained for the three plants of that particular group. The results are expressed as bar graphs plotted for "fold change" in the metabolite content of the group after 7th and 15th day of elicitation w.r.t the "Day 0" content of the same group. Fig. 11 indicates fold change in polysaccharide content.

The results indicate that the maximum fold change occurred in case of the plants treated with 100×10^{-3} mol SA (2.04), followed by 100×10^{-3} mol fructose (1.67) and then 100×10^{-3} mol NaCl (1.47). Although in case of field studies (*in vivo* elicitation) the change is not very high when compared to untreated group (1.19), still it may prove significant and beneficial when huge amount of biomass is taken into consideration. In this experiment also, SA has proven to be the best abiotic elicitor in our study, which increases the polysaccharide content 2 times more than the untreated group.

As indicated by Fig. 12, the phenolic content has increased the most in the case of plants treated with 100×10^{-3} mol SA (~8 times), followed by fructose (~5 times) and then NaCl (3.5 times). However, the change due to treatment is much more when compared to the untreated group which showed a fold change of 1.5 from 0th to 15th day. Furthermore, we could observe that the fold change has reduced considerably in plants under salt stress from 7 to day 15.

Thus, in case of *in vivo* elicitation, SA worked out to be the most potent abiotic elicitor, especially in the case of phenolics. The fold change in the metabolite concentration was significantly high in treated plants compared to untreated plants. The decrease in content of phenolic compounds from 7th to 15th day in case of NaCl-treated plants may be due to saline shock after initial stage, which led to poor growth of plants and flaccid leaves, observed at the end of the study. Due to lack of such short-term field elicitation studies, our study might prove quite fruitful for enhancing the metabolite production, and hence, the medicinal efficiency of the plant.

This has also been proven in previous studies where they have suggested that "SA has an important role in activation of the plant specific-Type III polyketide biosynthetic pathway." This pathway is responsible for

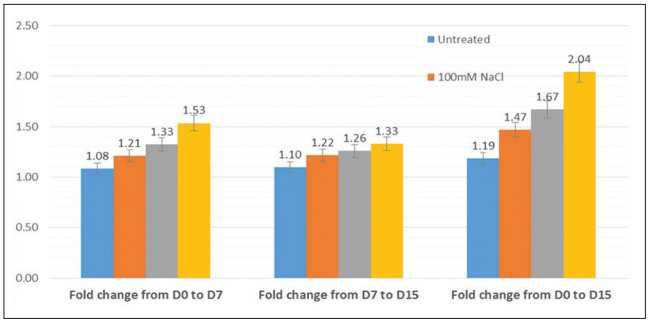

Fig. 11: Fold change in polysaccharide content from day 0 (D0) to day 7 (D7) and day 15 (D15) in untreated and treated (salt, sugar, and salicylic acid); p≥0.05

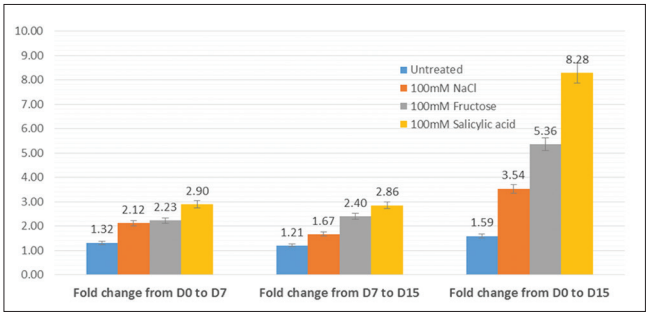

Fig. 12: Fold change in phenolic content from day 0 to day 7 and day 15 in untreated and treated (salt, sugar, and salicylic acid); p≥0.05

synthesis of tricyclic aromatic quinones and apart from this, SA also helps in the efficient utilization of carbon-sources, thereby enhancing polysaccharide production by other metabolic pathways [31]. It was also observed in our study that the rate of change is more up to 96 hrs of elicitation than later. This can be explained by studies that suggest that maximum physiological changes that occur due to any type of stress (biotic or abiotic) in a plant tissue happen during the first 72 hrs of inducing stress [32].

DISCUSSION

Earlier studies suggest successful use of auxin in the culture medium to elongate the shoot buds. Researchers reported the use of 2 mg/l IBA along with BA resulted in 100% response with elongation of 6–8 shoot buds per explant [31]. This was also confirmed by results of our study and fully organized multiple shoots were obtained by regulating the exogenously fed growth regulators.

Role of various growth regulators have been manifested in various recalcitrant plant cultivars. Studies have reported beneficial effects of BAP and IBA in C. annuum organogenesis [32-33]. Fully organized multiple shoots were obtained in C. *frutescens* by administering BAP. Both BA and IBA induced direct differentiation of shoot buds from cotyledonary leaf explants in C. *frutescens* [34-35]. Similar observations were made in Tagetes under influence of BA and IBA [35-36]. The PGRs added to the media act as elicitors for the metabolic pathways for the phytochemicals [30], hence, the regenerated plantlets were metabolically similar or superior to the parent plant.

The application of elicitors to enhance secondary metabolite production in plants, is like mimicking the production of these compounds naturally in the presence of pathogen infection. Plants usually develop a defensive response that activates a sequential reaction of complex multi-component network on pathogen attack. These usually include the production of secondary metabolite such as pheonolics. The overproduction of secondary metabolite in Capsicum sp. is already

well studied using in-vitro models [36-38]. The use of abiotic and biotic elicitors for the production of high capsaicinoid content using the cell suspension cultures is well documented. Effects of abiotic elicitor on flowering when sprayed on field grown plants, enhanced levels of phenyl propanoid compounds [39].

Plant defence can be triggered by local recognition of pathogens but, more effective responses include systemic signalling pathways [40]. Two of the most important compounds having this ability are salicylic acid (SA) and jasmonic acid (JA). Systemic responses include those dependent on SA signalling and are named Systemic Acquired Resistance [41]. The effect has also been proven in previous studies where they have suggested that "SA has an important role in activation of the plant specific-type III polyketide biosynthetic pathway." This pathway is responsible for synthesis of tricyclic aromatic quinones and apart from this, salicylic acid also helps in efficient utilization of carbon-sources, thereby enhancing polysaccharide production by other metabolic pathways [42].

It was also observed in our study that the rate of change is more up to 96 hrs of elicitation, than later. This can be explained by studies that suggest that, maximum physiological changes that occur due to any type of stress (Biotic or abiotic) in a plant tissue, happen during the first 72 hrs of inducing stress [43].

The Induced Systemic Resistance is known to be dependent on JA [44]. SA, JA and its derivatives like Methyl Jasmonate (MeJ) have been used as inducers in plants and were found to stimulate their secondary metabolism [45-46]. The ability of jasmonate to boost plant defences against fungal pathogens has already been reported [46]. The mechanism of action of SA and MeJ (or more general, jasmonates) is still a matter of debate [47]. These two compounds seem to act independently via antagonistic pathways giving rise to different plant responses. Nevertheless, a clear dichotomy does not always exist.

CONCLUSION

In the present investigation, micropropagation was done using young shoot tip, which gave the best result when cultured on MS media with 0.2×10^{-3} g/L IBA and 0.2×10^{-3} g/L BAP. 0.2 g/L of citric acid gave the best control of phenolic secretion during shoot proliferation. For shoot elongation and rooting, MS media with 2.0×10^{-3} g/L BAP and 0.2×10^{-3} g/L IBA gave best results. During rooting of explants, activated charcoal (2 g/L) was more successful in preventing browning of the media. The regenerated plants were morphologically similar to the parent plant and the metabolic profile (only polysaccharides and phenols) were also similar, rather slightly enhanced in regenerated plants.

Elicitation studies prove that SA can be an effective abiotic elicitor, for suspension cultures of *in vitro* grown callus. It increased the content of polysaccharides, up to 7 times and phenolic compounds to almost 10-fold compared to untreated control kept under same conditions for the same duration. *In vivo* elicitation studies also indicated that SA is a potent elicitor that enhances metabolite production. Although *in vivo,* the effect is less pronounced in case of polysaccharides (0.8 times) but quite high for phenolic compounds (~7 times) in comparison to the untreated group, after 15 days of elicitation. Fructose/sugars also have a significant effect on metabolite elicitation. Salt stress had minimal effect on enhancement of metabolite synthesis.

A. vera having wide array of bioactive constituents, being used as a medicinal product or for cosmetic purpose throughout the world is rightly called the "Miracle Plant." Due to recalcitrant and genotype specificity, regeneration and elicitation are the strong tailback.

REFERENCES

1. Rathor N, Arora T, Manocha S, Patil AN, Mediratta PK, Sharma KK. Anticonvulsant activity of *Aloe vera* leaf extract in acute and chronic models of epilepsy in mice. J Pharm Pharmacol 2014;66(3):477-85.
2. Sajjad A, Sajjad SS. *Aloe vera*: An Ancient herb for modern dentistry- A literature review. J Dent Surg 2014; DOI:10.1155/2014/210463.
3. Venkataramaiah V. *Aloe*: A key medicinal herb for drug and cosmetic Industry. Sapthgiri. Available from: http://www.tirumala.org/sapthgiri/012003/Aloe.htm. [Last cited on 2003].
4. Deng XH, Chen WX, He QM, Zhu LF. Utilization and resources protection of *Aloe vera* L. Var. Chinensis (Haw.) Berger. J Plant Resour Environ 1999;8:26-30.
5. Waller GR, Mangiafiaco S, Ritchey CR. A Chemical Investigation of *Aloe Barbadensis* Miller. Proceedings of the Oklahoma Academy of Science. Oklahoma, USA; 1978. p. 6976.
6. Abdi G, Hedayat M, Modarresi M. *In vitro* micropropagation of *Aloe vera* – Impacts of plant growth regulators, media and type of explants. J Biol Environ Sci 2013;7(19):19-24.
7. Boudreau MD, Beland FA. An evaluation of the biological and toxicological properties of *Aloe barbadensis* (miller), Aloe vera. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 2006;24(1):103-54.
8. Eshun K, He Q. *Aloe vera*: A valuable ingredient for the food, pharmaceutical and cosmetic industries – A review. Crit Rev Food Sci Nutr 2004;44(2):91-6.
9. He Q, Changhong L, Kojo E, Tian Z. Quality and safety assurance in the processing of *Aloe vera* gel juice. Food Control 2005;16(2):95-104.
10. Ni YW, Yates KM, Zarzycki R. Inventor. Aloe Pectins. United States Patent US 5929051; 1999.
11. Vinson JA, Al Kharrat H, Andreoli L. Effect of *Aloe vera* preparations on the human bioavailability of vitamins C and E. Phytomedicine 2005;12(10):760-5.
12. Sanchez IC, Natali L, Cavallini A. *In vitro* culture of *Aloe barbadensis* Mill. Morphogenetic ability and nuclear DNA content. Plant Sci 1988;55(1):53-9.
13. Bedini C, Caccia R, Triggiani D, Mazzucato A, Soressi GP, Tiezzi A. Micropropagation *of Aloe arborescens* Mill: A step towards efficient production of its valuable leaf extracts showing anti-proliferative activity on murine myeloma cells. Plant Biosyst 2009;143(2):233-40.
14. Sanchez IC, Natali L, Cavallini A. *In vitro* culture of *Aloe barbadensis* Mill: Micropropagation from vegetative meristems. Plant Cell Tiss Organ 1990;20(1):71-4.
15. Roy SC, Sarkar A. *In vitro* regeneration and micropropagation of *Aloe vera* L. Sci Hortic 1991;47(1):107-11.
16. Meyer HJ, Staden JV. Rapid *in vitro* propagation of *Aloe barbadensis* Mill. Plant Cell Tiss Organ 1991;26(3):167-71.
17. Corneanu M, Corneanu G, Vekas M, Minea R. *In vitro* organogenesis of *Aloe arborescencs* (*Liliaceae*). Rev Roum Biol Anim 1994;39(1):45-52.
18. Richwine AM, Tipton JL, Thompson A. Establishment of *Aloe, Gasteria,* and *Haworthia* shoot cultures from inflorescence explants. Hortscience 1995;30(7):1443-4.
19. Abrie A, Staden JV. Micropropagation of endangered *Aloe polyphylla*. Plant Growth Regul 2001;33(1):19-23.
20. Chaudhuri S, Mukundan U. *Aloe Vera* L. - Micropropagation and characterization of its gel. Phytomorphology 2001;51(2):155-7.
21. Groenewald EG, Koeleman A, Wessels DC. Callus formation and plant regeneration from seed tissue of *Aloe pretoriensis*. Z Pflanzenphysiol 1975;75:270-2.
22. Garro-Monge G, Gatica-Arias AM, Valdez-Melara M. Somatic embryogenesis, plant regeneration and acemannan detection in *Aloe* (*Aloe barbadensis* Mill.). Agron Costarric 2008;32(2):41-52.
23. Rathore MS, Chikara J, Shekhawat NS. Plantlet regeneration from callus cultures of selected genotype of *Aloe vera* L. - An ancient plant for modern herbal industries. Appl Biochem Biotechnol 2011;163(7):860-8.
24. Rodr´iguez-Garc´ia R, Rodr´iguez DJ, Gil-Mar´in JA, Angulo-S´anchez JL, Lira-Saldivar RH. Growth, stomatal resistance, and transpiration of *Aloe vera* under different soil water potentials. Ind Crops Prod 2007;25(2):123-8.
25. Yepez LM, Diaz ML, Granadillo E, Chacin F. Optimum frequency of irrigation and fertilizers application in *Aloe vera* L. Turrialba 1993;43(4):261-7.
26. Schaik AH, Van Struik PC, Damian TG. Effects of irrigation and N on the vegetative growth of *Aloe barbadensis* Mill-in Aruba. Trop Agric 1997;74(2):104-9.
27. Diaz M, Gonzalez A. Capacidad CAM en Zαbila: Respuesta a la intensidad de luz. Presented at Universidad Nacional Experimental Francisco de Miranda (UNEFM), Cieza, Venezuela; 1986.
28. Hansen J, Moller I. Percolation of starch and soluble carbohydrates from plant tissue for quantitative determination with anthrone. Anal

Biochem 1975;68(1):87-94.
29. Singleton VL, Orthofer R, Lamuela-Raventós RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Method Enzymol 1999;299(1):152-78.
30. Abdul Jaleel C, Gopi R, Gomathinayagam M, Panneerselvam R. Traditional and non-traditional plant growth regulators alters phytochemical constituents in *Catharanthus roseus*. Process Biochem 2009;44(2):205-9.
31. Husain S, Jain A, Kothari SL. Phenyl acetic acid improves bud elongation and *in vitro* plant regeneration efficiency in *Capsicum annum* L. Plant Cell Rep 1999;19:64–8.
32. Valera-Montero LL, Ochoa-Alejo N. A novel approach for chili pepper (*Capsicum annuum* L.) plant regeneration: shoot induction in rooted hypocotyls. Plant Sci 1992;84:215-9.
33. Kumar V, Gururaj HB, Narasimha Prasad BC, Giridhar P, Ravishankar GA. Direct shoot organogenesis on shoot apex from seedling explants of *Capsicum annuum* L. Sci Horti 2005;106:237-46.
34. Misra P, Datta SK. Direct differentiation of shoot buds in leaf segments of white marigold (*Tagetes erecta* L.). *In Vitro* Cell Dev Biol Plant 2001;37:466-70.
35. Chakrabarty R, Singh DT, Garg GK. Transformation studies on chickpea embryo axis. J Plant Biochem Biotechnol 2000;9:107-10.
36. Johnson TS, Ravishankar GA, Venkataraman L. Separation of capsaicin from phenyl propanoid compounds by high performance liquid chromatography to determine the biosynthetic status of cells and tissues of Capsicum frutescens Mill. *in vivo* and *in vitro*. J Agric Food Chem 1992;40:2461-63.
37. Johnson TS, Ravishankar GA. Precursor biotransformation in immobilized placental tissues of Capsicum frutescens Mill II. Influence of feeding intermediates of the capsaicinoid pathway in combination with L-valine on capsaicin and dihydrocapsaicin accumulation. J Plant Physiol 1996;153:240-3.
38. Rao RS, Ravishankar GA. Biotechnological production of Phytopharmaceuticals. J Biochem Mol Bio Biophys 2000;4:73-102.
39. Prasad Narasimha BC, Gururaj HB, Kumar V, Giridhar P, Parimalan R, Sharma A, Ravishankar GA. Influence of 8-Methyl-nonenoic acid on Capsaicin Biosynthesis in *In Vivo* and *In Vitro* Cell Cultures of Capsicum Spp. J Agric Food Chem 2006;54:1854-59.
40. Conrath U, Pieterse CMJ, Mauch-Mani B. Priming in plant–pathogen interactions. Trends Plant Sci 2002;7:210-16.
41. Dempsey DA, Shah J, Klessig DF. Salicylic acid and disease resistance in plants. Crit Rev Plant Sci 1999;18:547-75.
42. Wang B, Zha X. Effect of salicylic acid on cell growth and polysaccharide production in suspension cultures of protocorm-like bodies from Dendrobium huoshanense. Sheng Wu Gong Cheng Xue Bao. 2009;25(7):1062-8.
43. Liu F, Andersen MN, Jacobsen SE, Jensen CR. Stomatal control and water, use efficiency of soybean (*Glycine max* L. Merr.) during progressive soil drying. Environ Exp Bot 2005;54:33-40.
44. Feys BJ, Parker JE. Interplay of signaling pathways in plant disease resistance. Trends Genet 2000;16:449-55.
45. Hahlbrock K, Bednarek P, Ciolkowski I, Hamberger B, Heise A, Liedgens H, Logemann E, Nürnberger T, Somssich IE, Tan J. Non-self recognition, transcriptional reprogramming, and secondary metabolite accumulation during plant/pathogen interactions. Proc Natl Acad Sci USA 2003;25(100):14569-76.
46. Thomma B, Eggermont K, Brockaert W, Cammue B. Disease development of several fungi on Arabidopsis can be reduced by treatment with methyl jasmonate. Plant Physiol Biochem 2000;38:421-7.
47. Felton GW, Korth KL. Trade-offs between pathogen and herbivore resistance. Curr Opin Plant Biol 2000;3:309-14.