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Rooting effect of active charcoal for high scale micropropagation of stevia (*Stevia rebaudiana* Bertoni)

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Abstract

Stevia rebaudiana Bertoni is a perennial, herbaceous and open pollinated plant containing non-caloric and natural sweetener. The problem of this plant is culture restrictions due to its failed in seed germination and hollow seeds. The first step of rectify this problem is micro propagation procedure leads to produce healthy plants in large scale. In this work, the impact of Active charcoal on micro propagation of stevia was investigated. The used levels of Active charcoal were 0.0, 0.5, 1.0, 1.5 and 2.0 g/l which examined on nodal and shoot tip explants. Experiment was conducted in factorial based on completely randomized design (CRD) with 3 replications and observations was carried out after the 4 weeks. The nutritional requirements of explants were proved by Murashige & Skoog medium. The analysis of collected data statically (ANOVA) showed that using of charcoal in culture medium has significant difference on studied treats. The obtained results demonstrated that plant height, leaf number and the rate of rooting in explants have increased above 2.5, 2.5 and 4.0 folds respecting at the presence of 1.5 g/l charcoal.

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Introduction

Stevia rebaudiana Bertoni is a perennial, herbaceous and open pollinated plant containing non-caloric and natural sweetener. It is a member of the Asteraceae family (Jagatheeswari and Ranganathan, 2012). It is indigenous to the Rio Monday Valley of the Amambay Moutain Region where it grows as a perennial at an altitude between 200-500 meters having a mean temperature of 23-43˚C and rainfall ranging from 1500-1800 mm per annum. The native Guarani tribe had known for centuries the unique sweetening power of its leaves and other medicinal properties. They called the plant "kaa he-he" which translates as "sweet herb" and used it as sweetener for their green herbal tea"mate" and other domestic purposes as a flavourenhancer. In due course, it was introduced to settler (Din *et al.,* 2006).

Its leaves contain approximately 10% of steviosides which are intensely sweet compounds (150 to 300 times sweeter than sugar). The leaves have been traditionally used for hundreds of years in Paraguay and Brazil to sweeten local teas, medicines and as a 'sweet treat'. Japan is now the largest consumer of steviosides extracted from stevia leaves in Japan stevia replaces the chemical sweeteners, aspartame etc, which were banned there in the 1970's. Other countries use lesser quantities of steviosides. Steviosides have zero calories and can be used wherever sugar is used, including in baking etc (Madan *et al.,* 2010).

Stevia is the new emerging alternative source of calorie free sweetener having no carbohydrate and fat. It is 20 to 30 times sweet than cane and beet sugar, highly nutritious, delicious, non-toxic and non-additive sugar. It also enhances the flavour, helpful in digestion, weight reduction, anti oxidant, prevents dental caries and having antimicrobial and anti-plaque properties, increases mental alertness, increase energy levels but does not affect the blood sugar level, therefore key-source sweetener for diabetic world (Pande and Khetmalas, 2011). Besides, Stevia can be used in hypertension, hypoglycemic, helpful in skin toning and healing, tobacco and alcohol cravings and a tonic for pancreas. It can also be used as alternative source of sugar for food confectioneries, bakeries, fruit, juices, jams, biscuits, chocolatesl vegetables and other food stuffs. The recent researches along with future prospective of this new emerging medicinal plant. Stevia is a valuable medicinal plant species and it is being used for the treatment of diabetes. Currently, there is a high demand for raw material of this medicinal herb due to ever increasing diabetes disorder among the population (Goyal *et al.,* 2010).

Currently, stevia is being propagated by stem cuttings. Low seed germination percentage is a major limiting factor for large scale cultivation of stevia plant species for commercial usage. Further vegetative propagation is also limited by the less number of individuals obtained from single plant. Therefore, a suitable alternative method for large scale plant production within a short period is the use of in vitro culture technology (Thiyagarajan and Venkatachalam, 2012).

It seems that production of this plant would be increased due to specific uses and consumption demands. However, there are some barriers on this way. Lower germination percentage, along with small seed size are accounted as major restriction factors. Farther more, the research showed that this problem is mainly coused by producing hollow seeds. Also stevia is a self incompatibility plants and natural produce seeds would be genetic hetero zygote cousin variation in sweeteners. The conventional cutting method for propagation on this plant leads to develop on sufficient plants (Debnath, 2008).

To overcome such the problems many researchers used some additional agents like Active charcoal which has protective role rather than nutritional effect. Main reason of this work is the potential of Active charcoal in absorbing of phenol growth inhibitors. Production of these inhibitors in culture media by explants is a common for some plant species which cause damage on used the explants as well as deferential pathways (Tadhani *et al.,* 2006). For

instance in vinca in these plants the cutting of explants from main source leads to releasing polyphenol oxidase which develops darkness color at cutting faces. These compounds usually damage the explants and develop severe necrosis and inhibit the natural activate explants and most likely kill the cells. Browning tissues is result of tissue damages caused by polyphenoloxidase enzyme (Din *et al.,* 2006).

In order to study this problem and reduce browning, explants are cultured in culture media containing antioxidants including citric acid, ascorbic acid, mercapto ethanol. To rectify this on wanted evidence the use of in antioxidant is reverent job. Ascorbic acid, poly vinyl pyrrolidone, sodium diethyl dithio carbamate and Active charcoal are example of this count of agent among them Active charcoal is count for a cost effective than others (Pan and Van Staden, 2004).

Application of Active charcoal is not permanently useful so that it could absorb growth regulators, thiamine, nicotinic acid which are necessary for in vitro culture. The result of this negative effect leads to decreasing the available of mentioned compound in medium. Also addition of Active charcoal in medium has lowering effect on pH which substantially develops solid gelly condition (Van Waes, 1987). In this investigation were studied effect of Active charcoal on micro propagation of stevia was investigated.

Materials and methods

Plant material

Stevia rebaudiana Bertoni plants were procured from Agriculture Biotechnology Research Institute of Iran .In this experiment, shoot tip and node segments were used as explants.

Explant sterilization

The shoot tip and node explants were washed in tap water and gently rinsed with 20% (v/v) extra and surface sterilized in 0.1% sodium hypochlorite solution for 10 min and then rinsed with five changes of sterile distilled water.

Culture medium

The culture medium consisted of MS (Murashige and Skoog, 1962) salts, vitamins, 3% (w/v) sucrose and the pH of the medium was adjusted to 5.6 with 0.1 N NaOH or HCl before adding of 0.7% (w/v) agar. Culture medium sterilization were done with autoclaved at 121 ◦C for 15 min. The pH of the medium was adjusted to 5.8 before autoclaving.

Culture establishment

After surface sterilized shoot tip and node explants were cultured on MS medium supplemented with different concentrations of Active charcoal (0.0, 0.5, 1.0, 1.5 and 2.0 g/l) for micropropagation Stevia rebaudiana. The cultures were incubated at 24±2 ◦C under 16/8 h (light/dark cycle) photoperiod (60 µE m^{-2} s^{-1}) and irradiance provided by cool-white fluorescent tubes.

Statistical analysis

Experiments were done in factorial based on completely randomized design (CRD) with 3 replications and observations were recorded after the 4 weeks. The analysis of variance (ANOVA) was performed using SAS program. The differences among means were determined by Dunkan Test at 1% significant level.

Results and Discussion

Developing strong in vitro plants using node culture is necessary to provide plant materials for growing in green house and field conditions. The impact of different concentrations of Active charcoal(0.0, 0.5, 1.0, 1.5 and 2.0 g/l) were examined on growth vigor and rooting capacity of in vitro propagated plantlets derived from apical shoot and single node cultures. The statistical analysis of variation for shoot growth, leaf number and rooting rate per plant showed significant differences for all characters by all treatments except rooting rate for explants type.

Shoots length

Interactive source variation of Active charcoal× explants showed significance at $p<0.01$ for length of in vitro produced plantlets (Table 1). Results of shoot

length were illustrated on figure 1. As indicated on this figure, Although the highest lead of Active charcoal led to the longest shoot derived from node explants best this led of Active charcoal had inhibitory effect on plantlet growth derived from shoot tip explants.

Table 1. Analysis of variance (ANOVA) for different characters in Stevia rebaudiana L.

Source of variance	Degrees of freedom	Mean square		
		Shoots length	Leaf number	Root number
Active charcoal	4	5613/20**	43/00**	75/44**
Explant	1	1856/53**	13/33**	1.22ns
Active charcoal×Explant	4	73/36**	2.77**	17/13**
Error	20	3/22	0/86	1/85
CV٪		11/5	8/2	17/6

**: Significant at 1٪ probability level

ns: Non significant.

The best response by this explants were recorded at concentration 1.5 g/l activated charcoal. The differences between two explants type could be due to substantial vigor of shoot tip against node explants leading to develop strong plantlets, whilst high concentration of Active charcoal(2.0 g/l) adsorb required nutrient elements.

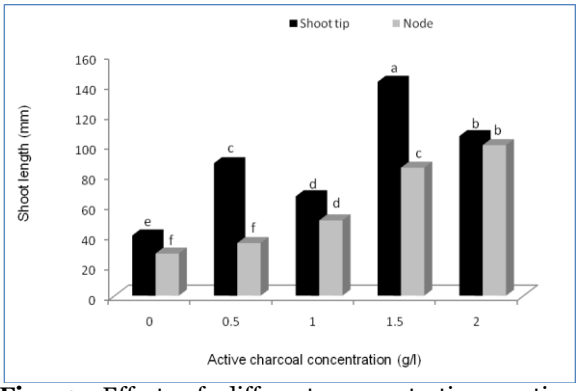

Fig. 1. Effect of different concentrations active charcoal on shoot length from different explants of stevia.

Leaf number pre plantlet

Apart from shoot length higher number of leaves in each plantlet is considered a useful trail from multiplying by invitro methods. Since, this character is differentially affected by different concentrations of Active charcoal in each explants type, comparison of analysis mean was conducted for interaction source of variable (figure 2). The best concentration of Active charcoal in this trait was 1.5 mg/l as conducted for shoot length to at shoot tip explants. But regarding node explants the best concentration was achieved from 2.0 g/l of activated charcoal.

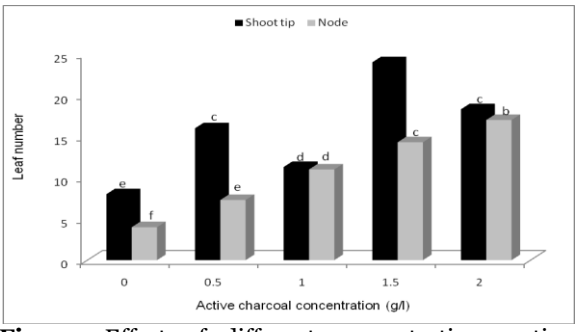

Fig. 2. Effect of different concentrations active charcoal on leaf number from different explants of stevia.

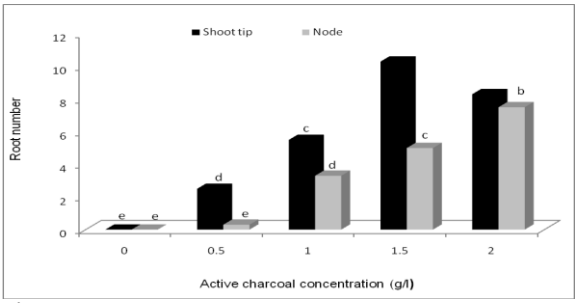

Fig. 3. Effect of different concentrations active charcoal on root number from different explants of stevia.

Root number

The effect of Active charcoal on rooting process of explants is more and similar to variations observed for shoot length and with the highest grade forconcentration of 1.5 and 2.5 g/l Active charcoal for

shoot tip and node explants respectively (figure 3).

Conclusion

The use of a potent inhibitor of poly phenol oxidase activity, Active charcoal in stevia micropropagation is strongly recommended as a major out were of this work showing 300٪ increase for shoot length and leave number at 1.5 g/l from shoot tip explants.

The rate of this promotion was recorded ~200٪ for node explants as well. Regarding root number, use of Active charcoal a critical so that control plantlet did not show any rooting activity in media lacking this agent. Since this phenomenon is necessary to transferring plantlets from invitro conducted to greenhouse/open field, apply a low cost agent in media used for micropropagation of stevia. This is accounted a cast effective method to produce high number of rooted plantlets without using any root inducing growth regulators.

Furthermore, Active charcoal containing media produced much healthier and stronger plantlets. Also, this media were enhancing support the plantlets for long time incubate at invitro condition (about 12 months). It seems that media containing activated charcoal, is continuously deactivating any potentially anti growth/ rooting compounds produced by stevia explant and plantlets. From economic point of view by this method each shoot tip and node explants are able to produce 24 and 18 nodes/leaf at a period of 30 days which could be repeatable by chain cycling to efficiently support the materials need for industrial scale produce of stevia and derivative compounds.

Acknowledgements

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