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Full Length Research Paper

An efficient callus initiation and direct regeneration of *Stevia rebaudiana*

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The experiment was carried out to establish an efficient callus induction system of *Stevia rebaudiana* from a variety of explants as well as direct regeneration from nodes. Callus initiation was done to extract secondary metabolites. However direct regeneration is essential for rapid large-scale clonal propagation. Among leaf, node and inter-nodal explants, the best callus initiation performance was observed by nodes. MS medium fortified with α-naphthalene acetic acid (NAA) 2.0 mg/L + 6-benzyladenine (BA) 2.0 mg/L showed the highest (93.33 ± 6.67%) callus induction by nodal explants. Though inter-nodal explants showed a moderate response (73.33 ± 6.67%) for callus induction in MS medium complemented with NAA 3.0 mg/L + BA 1.0 mg/L, nodal explants showed higher response (86.67 ± 13.33%) than inter-nodal explants in that nutritional environment. Leaf explants always showed very poor callus. However, the best direct regenerating medium was MS medium + BA 1.0 mg/L for multiple shoot proliferation and then transferring those shoots to ½ MS medium + NAA 1.0 mg/L for root formation.

Key words: *In vitro*, *Stevia rebaudiana*, 2,4-D, α-naphthalene acetic acid (NAA), 6-benzyladenine (BA), plant growth regulators.

INTRODUCTION

Stevia rebaudiana belongs to the family Asteraceae, commonly known as sweet leaf, sugar-leaf, or simply stevia. The native of *S. rebaudiana* is in South America (Alhady, 2011), but now it is grown all over the world. It is one of the most valuable medicinal plant and widely grown for its sweet taste to use as an alternative for sugar. As a sweetener and sugar substitute, stevia's taste has a slower onset and longer duration than that of sugar. The leaves of *S. rebaudiana* contains diterpene glycosides, such as steviolbioside, rubsoside, rebaudioside A, B, C, D, E and F, dulcoside and stevioside (Starratt et al., 2002). The green powder of stevia-leaf has sweetness 20 to 25 times greater than sugar while the pure extract of stevia that is, stevioside is 300 times sweeter than cane sugar (Liu and Lee, 1995; Banerjee and Sarkar, 2008; Anbazhagan et al., 2010).

Due to the non-caloric and sweetening properties, stevioside has garnered attention with the rise in demand for low-carbohydrate, and low-sugar food alternatives (Kalpana et al., 2009). Stevia also has a strong antimicrobial activity. In addition to the sweetening and antimicrobial properties, it has therapeutic values such as antihyperglycemic, antihypersensitive agent (Chan et al., 1998; Jeppensen et al., 2002), contraceptive properties (Melis, 1999) and prevention of dental caries. In some countries, it has been available as a sweetener for decades or centuries; for example, stevia is widely used as a sweetener in Japan, China, Brazil, Indonesia, Tanzania and Korea (Goenadi, 1983; Brandle and Rosa, 1992; Fors, 1995).

A study reporting that steviol may be a mutagen has been criticized on procedural grounds that the data were mishandled in such a way that even distilled water would appear mutagenic. More recent studies appear to establish the safety of steviol and its glycosides. There are primarily two methods for the multiplication of stevia since seed germination of stevia is often poor (Miyazaki and Wantenabe, 1974; Goettemoeller and Ching, 1999;

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Sivaram and Mukundan, 2003). The first and most efficient method is *in vitro* propagation and second method is the stem cutting. The main drawback of stem cutting is that it is sometimes more expensive to produce than the tissue culture since the success rate of stem cutting is very low (Rafiq et al., 2007; Seema et al., 2011).

Due to the problems related with stem cutting, the necessity for agro-biotechnological approaches for *in vitro* propagation of stevia has been raised (Janarthanam et al., 2009). Thousands of people in Bangladesh are suffering from diabetic mellitus and high blood pressure. So stevioside may play a very important role in the treatment of this people (Uddin et al., 2006). For these reasons, the present investigation was undertaken to find out the suitable explant source with the best suited concentration of plant growth regulators for mass propagation of *S. rebaudiana* through *in vitro* technique.

MATERIALS AND METHODS

This research project was conducted at the plant genetic engineering lab, Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology (SUST), Sylhet-3114, Bangladesh.

Collection of explant

The young stevia plants were collected from BRAC, Gazipur, Dhaka, Bangladesh and grown in the pot. Different types of explants were prepared from those young plants. The types of explants were leaf, node and inter-node. However the size of leaf explants was about 1 cm^2 whereas the size of nodal and inter-nodal explants was about 3 cm.

Explant sterilization

The explants were thoroughly washed with distilled water for three times. Then, the explants were washed with 70% ethanol (for 2 to 3 min). In the laminar air flow cabinet, the explants were treated with 0.1% $HgCl_2$ with the addition of few drops of Tween-20 for inner surface sterilization (for 4 to 6 min). Finally, the explants were washed with sterile distilled water for several times to remove all the sterilizing agents and then the explants were cut into small pieces by sterile surgical blade ranging in size from 1.0 to 1.5 cm long.

Callus induction media

MS media (Murashige and Skoog, 1962) supplemented with 2,4-D varying in concentration from 1.0 to 4.0 mg/L were prepared separately in conical flasks. On the other hand, MS basal media supplemented with NAA + BA varying in concentration from 1.0 to 3.0 mg/L in different combination were prepared separately in different conical flasks. The 3% sucrose was added to each conical flask. The pH was adjusted to 5.6 to 5.8. Then, 0.7% agar was added for solidification of medium.

Multiple shoot formation media

Shoot regeneration media were prepared by mixing all the components as the callus induction media except hormones. BA varying in concentration from 1.0 to 3.0 mg/L was used. Explants were inoculated and data were recorded.

Inoculation of explant

Each explants were inoculated in each test-tube by the help of a sterile forceps in the laminar air flow chamber. After the inoculation of explants, the test-tubes were transferred to the culture room and incubated at 25°C. The photoperiod was maintained as 16 h light and 8 h darkness by 40 W white florescent tubes light with intensity from 2000 to 3000 lux. Data was recorded every week for three months.

Rooting media

We studied different strength of MS basal media supplemented with NAA for root formation. NAA varying in concentrations from 1.0 to 3.0 mg/L with MS basal medium strength of 1, ½, $^1/_3$ were prepared and inoculated with good shoot formed plantlets.

Statistical analysis

The data were recorded at regular interval for statistical analysis. Arithmetic mean (A.M.) and standard deviation (S.D.) were evaluated by analyzing data with Microsoft excel 2007. Standard error (S.E.) was deducted by dividing standard deviation by square root of the total number of the replication.

RESULTS AND DISCUSSION

Callus induction

The highest (93.33 ± 6.67%) callus induction (Figure 1) was obtained from nodal explants in MS medium fortified with NAA 2.0 mg/L + BA 2.0 mg/L. Leaves showed very poor callus than inter-nodal and nodal explants. Though inter-nodal explants showed its best (73.33 ± 6.67%) performance in MS medium + NAA 3.0 mg/L + BA 1.0 mg/L, nodal explants showed more (86.67 ± 13.33%) vigorous callus in that nutritional environment. Development and growth of calli varied with the types of explants. The responses of calli development from nodal explants were significantly higher than the leaf and inter-nodal explants. The leaf explants showed very poor calli when compared with inter-nodal and nodal explants. In the present study, MS medium fortified with NAA + BA was found to be the best hormonal concentrations for callus induction by inter-nodal and nodal explants. Though inter-nodal explants had its best response in MS medium complemented with NAA 3.0 mg/L + BA 1.0 mg/L, nodal explants exhibited higher callus induction in that environment if standard error of the arithmetic mean of the percent callus induction was ignored.

Nodal explants showed significantly higher response than leaf and inter-nodal explants in other treatments also. Therefore among leaf, nodal and inter-nodal explants nodes were considered as the best source of explants for *in vitro* callus culture of *S. rebaudiana* and

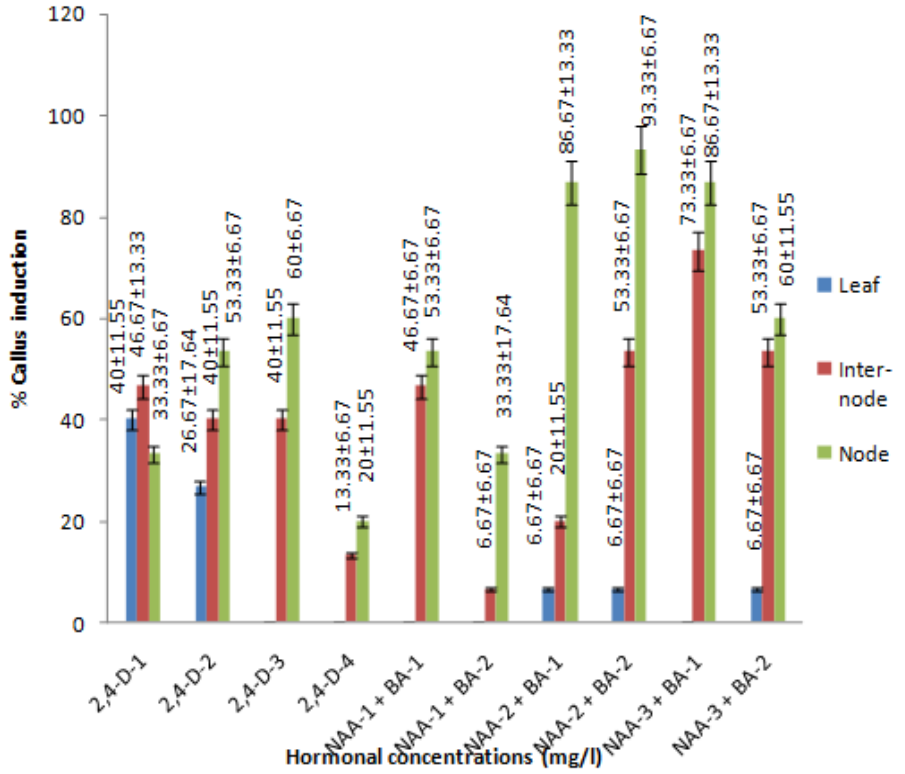

Figure 1. Effect of 2,4-D and NAA +BA in different concentration on callus induction by leaf, inter-node and node in MS media.

MS basal medium complemented with NAA 2.0 mg/L + BA 2.0 mg/L was regarded as the best nutrient medium for callus induction by nodal explants. Ali et al. (2010) also reported that NAA + BA for callus induction is by nodes. Though several authors reported 2,4-D for callus induction (Uddin et al., 2006; Janarthanam et al., 2010), we found this hormone less effective for callus induction of *S. rebaudiana* in comparison with NAA + BA. However, the calli induced by leaves were loosely arranged and friable while the calli induced by nodal explants was yellowish and non-friable (Figure 2). The obtained calli can be used for secondary metabolite extraction (Swanson et al., 2004; Janarthanam et al., 2010). But in this study, due to the limitation of some facilities, we were unable to study the extraction of steviol glycosides from callus. However, further study is required to increase the quantity of steviol glycosides accumulation in calli and establishment of rapid extraction process from calli.

Direct regeneration from inter-nodes

Multiple shoot formation

Multiple shoot proliferation from node was studied in MS basal media supplemented with various concentration of BA. The best shoot proliferating medium was MS basal medium containing BA 1.0 mg/L; showed 93.33 ± 6.67% response (Figure 3). Direct shoot multiplication has commercial value due to the rapid propagation of plantlets. Several authors also reported direct regeneration from explants (Ahmed et al., 2007; Satpathy and Das, 2010; Kalpana et al., 2010; Thiyagarajan and Venkatachalam, 2012). Ali et al. (2010) reported 90% shoot proliferating response from apical meristem in MS medium + BA 1.0 mg/L. Slavova et al. (2003) also used only BA for shoot formation of *S. rebaudiana*. In fact BA is a universal cytokine for shoot proliferation of most of the plants *in vitro*. However, MS basal media containing BA 2.0 and 3.0 mg/L had also very good response (86.67 ± 6.67 and 73.33 ± 6.67%, respectively) but lower hormonal concentration is more economic for large scale propagation. Therefore, MS medium + BA 1.0 mg/L was considered as the best. However, regeneration from nodal explants in MS medium often showed multiple shoot formation (Figure 4).

Root formation

The highest response of 86.67 ± 6.67% rooting (Figure 5) was found in ½ strength of MS basal medium supplemented with NAA 1.0 mg/L. Half strength of MS medium was more promising than full strength in the

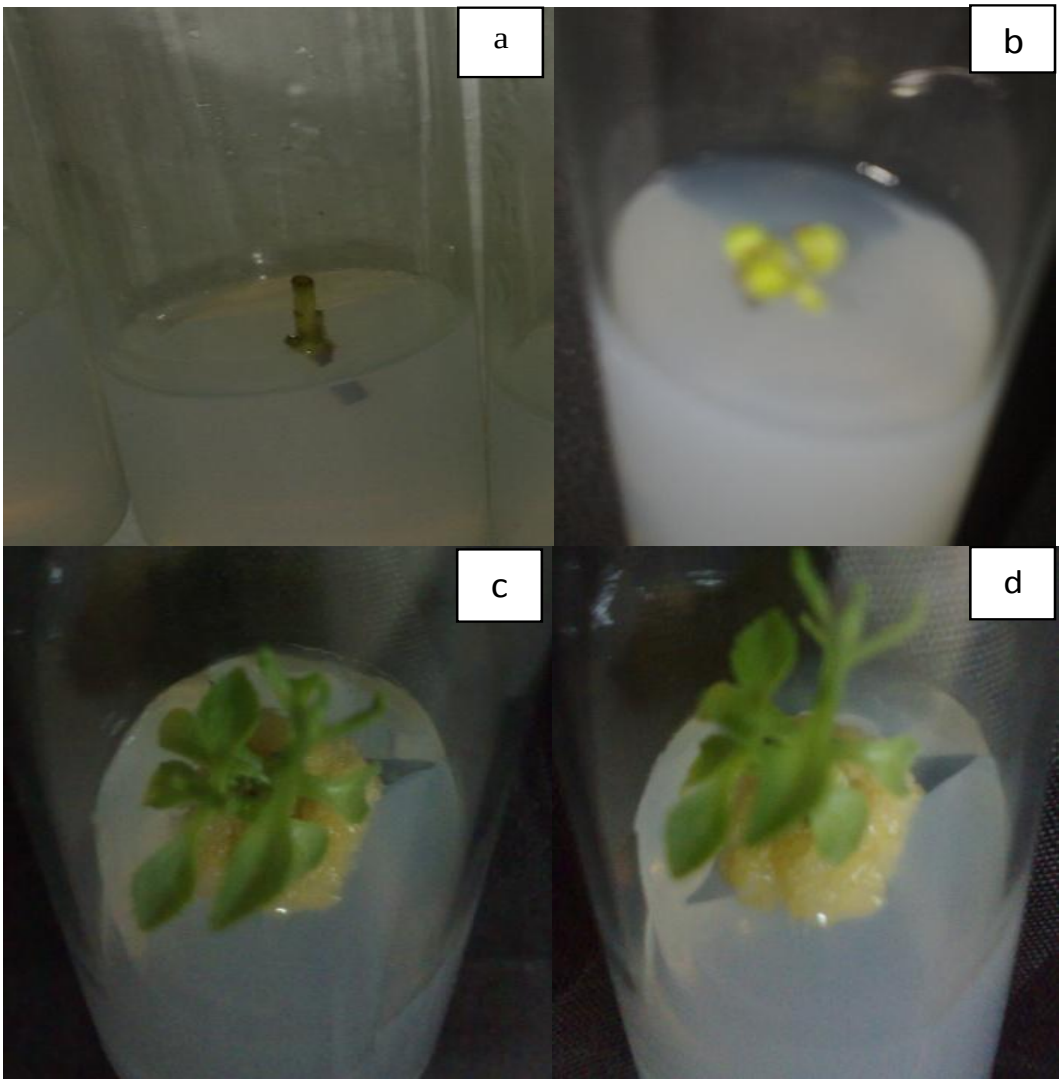

Figure 2. Callus induction stages by nodal explant in MS basal medium supplemented with NAA 2.0 mg/L + BA 2.0 mg/L. a, explant (node) inoculation. b, callus inducing response in two weeks; c, callus initiation after three weeks; d, profound callus after four weeks.

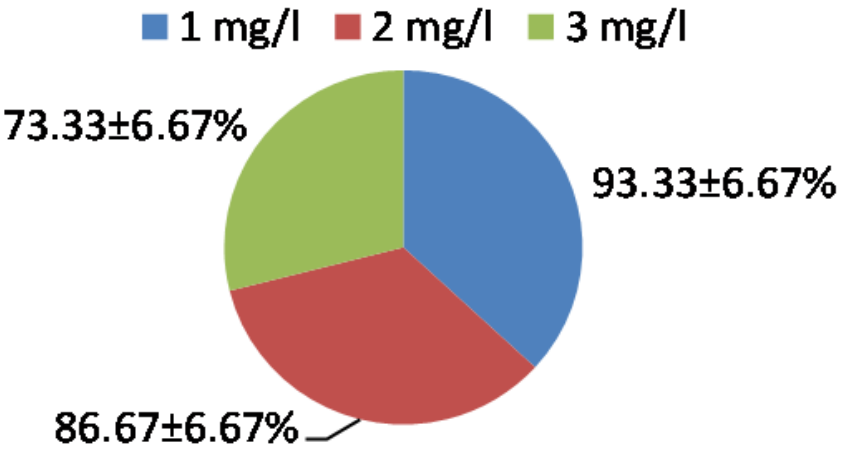

Figure 3. Effect of BA on shoot proliferation in MS media after two weeks.

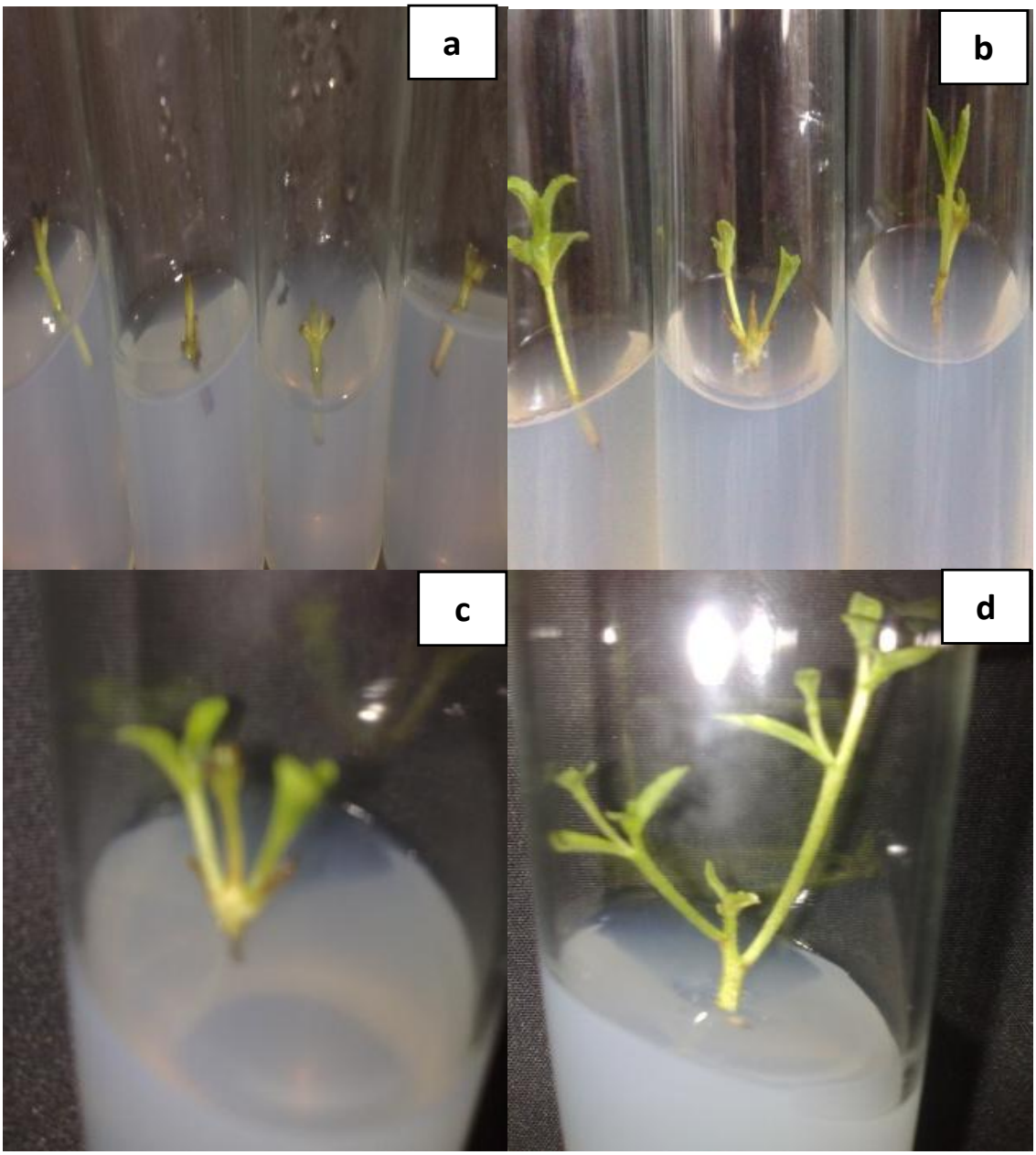

Figure 4. Direct shoot proliferation of *S. rebaudiana* from inter-nodes in MS medium fortified with 1.0 mg/L BA. a, explants (inter-nodes) inoculation; b, multiple shoot formation after two weeks; c, d, shoots proliferation after three weeks.

case of root formation of *S. rebaudiana*. In every hormonal treatment, half strength of MS basal medium showed better root formation response than that of full strength (100%) and one-third strength (33.33%). Sairkar et al. (2009) reported the best result for root formation by using half strength of MS media containing activated charcoal. Slavova et al. (2003) obtained 84 to 99% rooting in MS medium supplemented with NAA. Therefore, half (½) strength of MS basal medium containing NAA 1.0 mg/L was considered as the best nutritional environment for rooting of *S. rebaudiana.*

Conclusion

Among leaf, nodal and inter-nodal explants, the best source of explants for micro-propagation of *S. rebaudiana* is nodes. Nodes should be cultured aseptically *in vitro* in MS basal medium supplemented with NAA 2.0 mg/L + BA 2.0 mg/L for callus induction. If nodal explants are

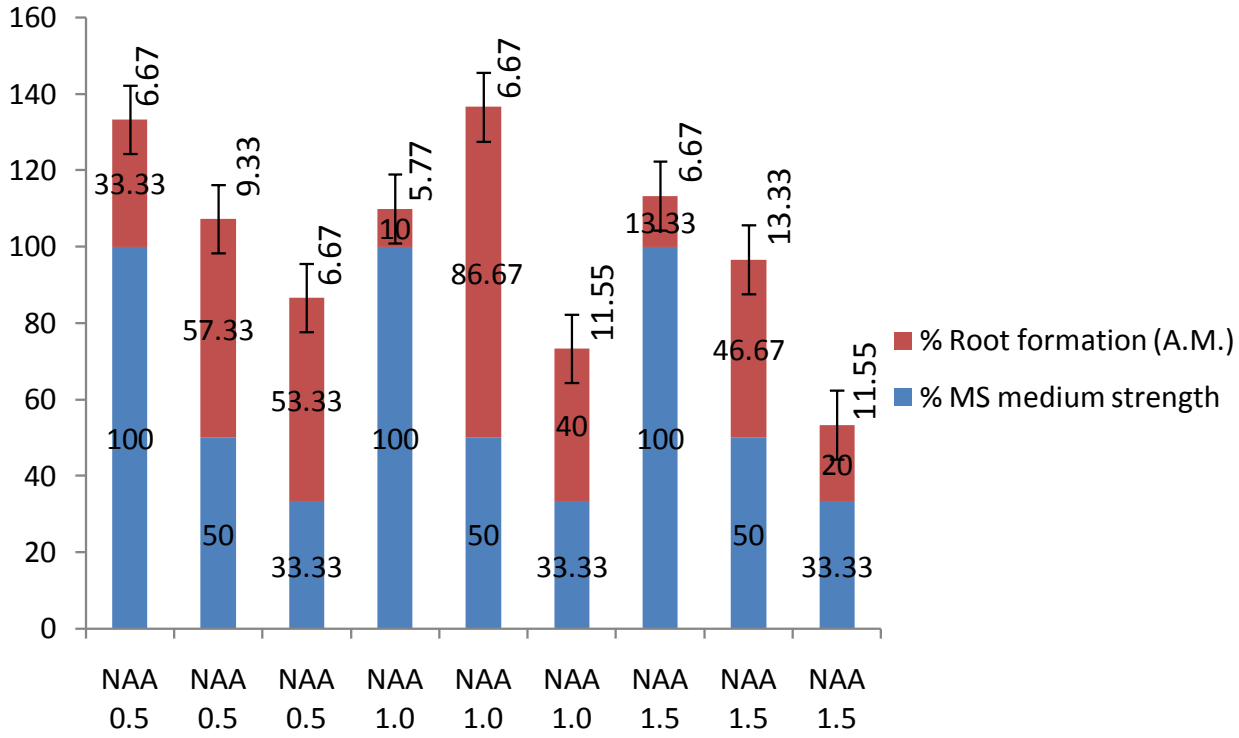

Figure 5. Root formation by different concentration of NAA in different strength of MS basal medium (full strength, 1/2 strength and 1/3 strength). The best (86.67 ± 6.67%) root forming medium was the ½ strength of MS medium supplemented with NAA 1mg/L.

short in supply, inter-nodal explants also can be used in MS basal medium containing NAA 3.0 mg/L + BA 1.0 mg/L. Calli of *S. rebaudiana* can be used for steviol glycoside extraction. However, direct regeneration from nodal explants for large-scale production can be done in MS medium with BA 1.0 mg/l for shoot proliferation and then transferring the shoots to ½ strength of MS basal medium containing NAA 1.0 mg/L.

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