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Comparison of various explants on callogenesis in *Stevia Rebaudiana*

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An experiment titled "Comparison of various Explants on Callogenesis in *Stevia rebaudiana*" was conducted in the tissue culture lab of Biotechnology at Nuclear Institute for Food and Agriculture (NIFA) during 2019. The experiment was carried out using Complete Randomized Block Design (CRBD) with three replications. The Murashige & Skoog basal (MS) media was supplemented with polyamines (PAs) in combination with plant growth regulators (PGRs) which were used for micropropagation of *Stevia*. Twelve different combinations of PAs and PGRs were used for callus induction. Data showed that different parameters such as Callus clour, callus texture were significantly affected. Observation of callus cells from different hormonal treatments showed that the cells morphology was significantly affected by the hormonal combinations. The best callogenic response was shown in leaf, as leaf segment produced large size callus as compared to internode segment. The morphology of calli formed from different explants were examined, and it was found that the calli obtained from leaves were mostly globular and green while that of internode were whitish and spindle shaped. The callogenic response was more pronounced in the presence of 2, 4-Dichlorophenoxy acetic acid (2, 4-D), Benzylaminopurine (BAP) and Putrescine (Put).

Keywords: *Stevia rebaudiana*, Callogenesis, Explants, Micropropagation.

INTRODUCTION

All life depends on plants. There is nothing more important in Plant sciences than to sustain and improve the green cover on earth. And for this, we have to learn how to protect plants, breed new varieties, and multiply them. We could do without missiles and bombs, and indeed even without much of modern technology, but not without plants Purohit (2013). *Stevia* is a member of Daisy family, generally recognized by the Indians who named it káahêê ("sweet herb"). *Stevia* is an elevated, taproot & having characteristics of herb Magalhaes et al. (2000). *Stevia* is determined by nature containing a lot of sugar and existing in all parts of the world and generally recognized as sweet leaf, sweet weed, sweet herb, methi patti and honey leaf. This plant is approximately calculated to be three hundred times sweeter than sugar cane while dry leaves are three hundred times sweeter than sugar Mehta et al. (2012*). Stevia* contains a high percentage of flavonoids, phenols & antioxidant activity Taleie (2012). Seed germination in *Stevia rebaudiana* is fruitless commonly due to infertile seed Kumar. (2013) and minute endosperm Yadav et al. (2011). *Stevia* seeds exhibit a very less regeneration percentage less than 10% Felippe et al. (1971). Almost eighty percent of the world population used medicinal plants as medicine. A large number of human populations have polygenic disease characterized by high glucose level in the blood commonly known as

diabetes. *Stevia rebaudiana* is used as a drug & relieve diabetes. Altaf et al. (2013). According to Thiyagarajan & Venkatchalan (2012). It is expected that in the year of 2025 fifty- seven million people would be affected by diabetes. It was further reported that the extracts of *Stevia* have no side effects & can be used as an alternative to sugar. The *Stevia* plant is a good choice for the currently demanding food supplements having low calories, low amount of carbohydrates & less sugar contents. *Stevia* plant has more medicinal benefits besides of its non-caloric properties. It is used to cure certain diseases like cancer Yasukawa et al. (2002), adiposity Dyrskog et al. (2005). The extracts of *Stevia* leaves are used for weight loss because it has the properties to reduce the desire for sweet foods. Hence, *Stevia* has significant value in medical & industrial field, its large scale production is necessary to get maximum benefit out of it. Jain et al. (2007). It regulates insulin & blood glucose level. Anton et al. (2010). The world's population is expanding immensely the available land for cultivation & plant growth should be utilized effectively to fulfill the needs of human beings. With the advancement of new technologies and capability of plants to develop into complete organism and for its rejuvenation plant tissue culture technique is introduced. Sakaguchi & Kan (1982). The objectives of this study were (i) to prepare protocols for micro propagation of disease free plants of *Stevia rebaudiana* for introduction in Pakistan. (ii) To examine the morphological traits of the callus (*Stevia*). .

MATERIALS AND METHODS

2.1 Experimental site and conditions:

This present research work on "Comparision of various Explants on Callogenesis in *Stevia rebaudiana*" was carried out in collaboration with Plant Breeding Management Group of Nuclear Institute for Food and Agriculture (NIFA), Peshawar during 2019. This study involved callogenesis of *Stevia* plantlets by optimizing conditions using various Plant Growth Regulators (PGRs) and polyamines (PAs) for its callogenesis. Two types of explants (leaf & internode) were used to determine better treatment for callus induction. Explants from the stored materials were kept on the regeneration (MS) medium (1962) incorporated with different doses of PGRs solitary as well as in combined form with polyamines. This research work was carried out under three different experiments and the experiments were carried using a Completely Randomized Design.

2.2 Surface sterilization

Young Leaf and internode cuttings as explants were initially washed with tap water for 15 mins to eradicate dust particles. Aseptic transfer of explants was cultured in a laminar flow bench. Before culturing the laminar flow bench was cleaned with 70% Ethanol & 95% Ethyl alcohol. Surface sterilization was done by immersion of explants on 70 % (v/v) Ethanol for 1 min & then in 0.2 (w/v) Mercuric Chloride ($HgCl_2$) solutions for 30 sec, followed by rinsing three times with autoclaved distilled water.

2.3 Aseptic transfer of explants

After sterilization, the explants were excised out in sterilized Petri plates with the help of sterilized forceps and cutting blades. The explants were then cultured in 100ml jars containing 25-35 ml of cultured medium. Finally, in the laminar air flow chamber, the explants were cut into small pieces ranging in size from 0.5 to 1.0 cm long. MS medium (Murashige & skoog 1962) was thoroughly used in the tissue culture experiment. Best media escaped from any contamination was selected for inoculation. Explants were successfully cultured aseptically on MS as basic medium incorporated with 3 % sucrose and provided with 7.0 % agar. Each explant was inoculated to MS medium with different concentrations of hormones. For callus initiation, sound leaf and internode segments were excised into 3-4 mm^2 cuttings for insertion on the media. For callogenesis various concentrations and combinations of PGRs and Polyamines were added to (MS) medium. MS0 (MS-medium without PGRs & PAs) was taken as a control. The culture vessels were placed in a 16 hours photoperiod provided by a white fluorescent lamp at $25^{\circ}C \pm 2^{\circ}C$. Data on different parameters was recorded after explants culturing in each experiment.

RESULTS AND DISCUSSION

3.1 Callus induction from leaf and internode segment:

The first step for callus induction was the selection of explants i.e. leaf and internode under aseptic condition. Leaf and internode segments as explants were inoculated on MS (Murashige and Skoog) media incorporated with selected Plant Growth regulators and polyamines combination. A

total of 12 treatments (T1-T12) were applied in different concentration for callus induction. The result showed promising influence of Plant Growth Regulators and Polyamines on callus induction. Best calluses were observed when explants were inoculated on MS medium Combined with BAP, Putrescine & 2, 4-D. The morophology of calli formed from different explants were investigated and it was found that the calli obtained from leaves were mostly globular and green while that of internode were whitish and spindle in shaped. Microscopic examination of callus cells from different hormonal treatments showed that the cells morphology was significantly affected by the hormonal combinations. The best callogenic response was shown in leaf as leaf segments produced more calli as compared to internode segment. While (MS0) medium without PGRs & PAs induced 0% callus (in control). Similarly medium containing combination of BAP (2mg/L) & Putrescine (1mg/L) induced shooting in leaf and internode explant. Gupta et al. (2010) examined the morphology of the calli prevailed from various explants and it was found that those obtained from root and leaf explants were shining green while, with nodal explant was found to be brown and hard. Furthermore, it was reported that the leaf explants could function as a good plant material for callus formation. Nasircilar et al. (2006) also stated that 2, 4-D produced earlier callus response than other Phyto regulators in barley, wheat & datura respectively. They also documented that 2, 4-D alone or in formulation with other phytoregulators is the main callus stimulating factor in plants. Karimian et al. (2014) stated that the combination & concentrations of phytoregulators play a crucial role in the growth and callus initiation.

Callus color:

Twelve different treatments were incorporated in Murashige and skoog (MS) basal media for callus colors. Various colors were observed.

Table 1: Effect of PAs and PGRs on the callus color of internode.

Treatments	Media concentration (mg^{-L})	Callus color
1	BAP (1) + Put (1)	Whitish
2	BAP (2) + Put (1)	Shoot / no callus
3	2, 4-D (1) + Put (1)	Whitish
4	2, 4-D (1.5) + Put (1)	Whitish
5	2, 4-D (2) + Put (1)	Whitish green
6	2, 4-D (1) + Put (1) + BAP (1)	Whitish
7	2, 4-D (1.5) + Put (1) + BAP (1)	Whitish yellow
8	2, 4-D (2) + Put (1) + BAP (1)	Whitish green
9	2, 4-D (1) + Put (1) + BAP (2)	Whitish
10	2, 4-D (1.5) Put (1) + BAP (2)	Whitish light green
11	2, 4-D (2) + Put (1) + BAP (2)	Whitish
MS	Control	No callus

Table 2: Effect of PAs and PGRs on the callus color of leaf .

Treatments	Media concentration (mg^{-L})	Callus color
1	BAP (1) + Put(1)	Dark green
2	BAP (2) + Put (1)	Yellowish + shoot
3	2, 4-D (1) + Put (1)	Yellowish white
4	2, 4-D (1.5) + Put (1)	Whitish
5	2, 4-D (2) + Put (1)	Yellowish
6	2, 4-D (1) + Put (1) + BAP (1)	Yellowish green
7	2, 4-D (1.5) + Put (1) + BAP (1)	Whitish green
8	2, 4-D (2) + Put (1) + BAP (1)	Whitish green
9	2, 4-D (1) + Put (1) + BAP (2)	Whitish green
10	2, 4-D (1.5) + Put (1) + BAP (2)	Yellowish
11	2, 4-D (2) + Put (1) + BAP (2)	Whitish light green
MS	Control	NA / No callus

Callus Morphology:

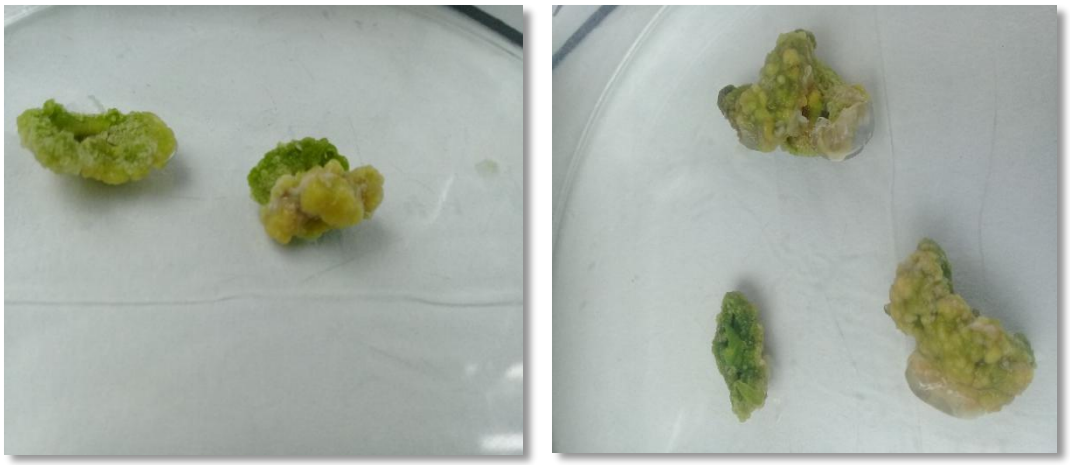
Twelve different treatments were incorporated in Murashige and Skoog (MS) basal media for callus texture. Different morphologies were examined on various concentration & combination of plant Growth Regulators (PGRs) and Polyamines (PAs) are presented in the table.

Table 3: Effect of polyamines and Plant Growth regulators on callus morphology of *Stevia* internode.

Treatments	Media concentration (mg^{-L})	Callus morphology
1	BAP (1) + Put (1)	Compact +(shooting)
2	BAP (2) + Put (1)	Shooting (No callus)
3	2, 4-D (1) + Put (1)	Clump and Spongy
4	2, 4-D (1.5) + Put (1)	Spongy and granular
5	2, 4-D (2) + Put (1)	Soft and clump
6	2, 4-D (1) + Put (1) + BAP (1)	Compact
7	2, 4-D (1.5) + Put (1) + BAP (1)	Hard
8	2, 4-D (2) + Put (1) + BAP (1)	Hard
9	2, 4-D (1) + Put (1) + BAP (2)	Hard
10	2, 4-D (1.5) + Put (1) + BAP (2)	Friable
11	2, 4-D (2) + Put (1) + BAP (2)	Hard and clump
MS	Control	Shooting (No callus)

Table 4 :Effect of polyamines and PGRs on callus morphology of *Stevia* leaf

Treatments	Media concentration (mg^{-L})	Callus morphology
1	BAP (1) + Put (1)	Compact
2	BAP (2) + Put (1)	shooting
3	2, 4-D (1) + Put (1)	Soft granular
4	2, 4-D (1.5) + Put (1)	Hard
5	2, 4-D (2) + Put (1)	Hard
6	2, 4-D (1) + Put (1) + BAP (1)	Soft
7	2, 4-D (1.5) + Put (1) + BAP (1)	Soft
8	2, 4-D (2) + Put (1) + BAP (1)	Hard / compact
9	2, 4-D (1) + Put (1) + BAP (2)	Hard / compact
10	2, 4-D (1.5) + Put (1) + BAP (2)	Soft granular
11	2, 4-D (2) + Put (1) + BAP (2)	Compact
MS	Control	NA / No callus



T8 (2mgL^{-1} 2, 4-D+1mgL^{-1}put & BAP)

T11 (2mgL^{-1} 2, 4-D+1mgL^{-1}put +2mgL-^{1}BAP)

T1 (BAP (1 mgL^{-1} + Put mg L^{-1})

Fig 1 : Callogenic response of Leaf Segments.

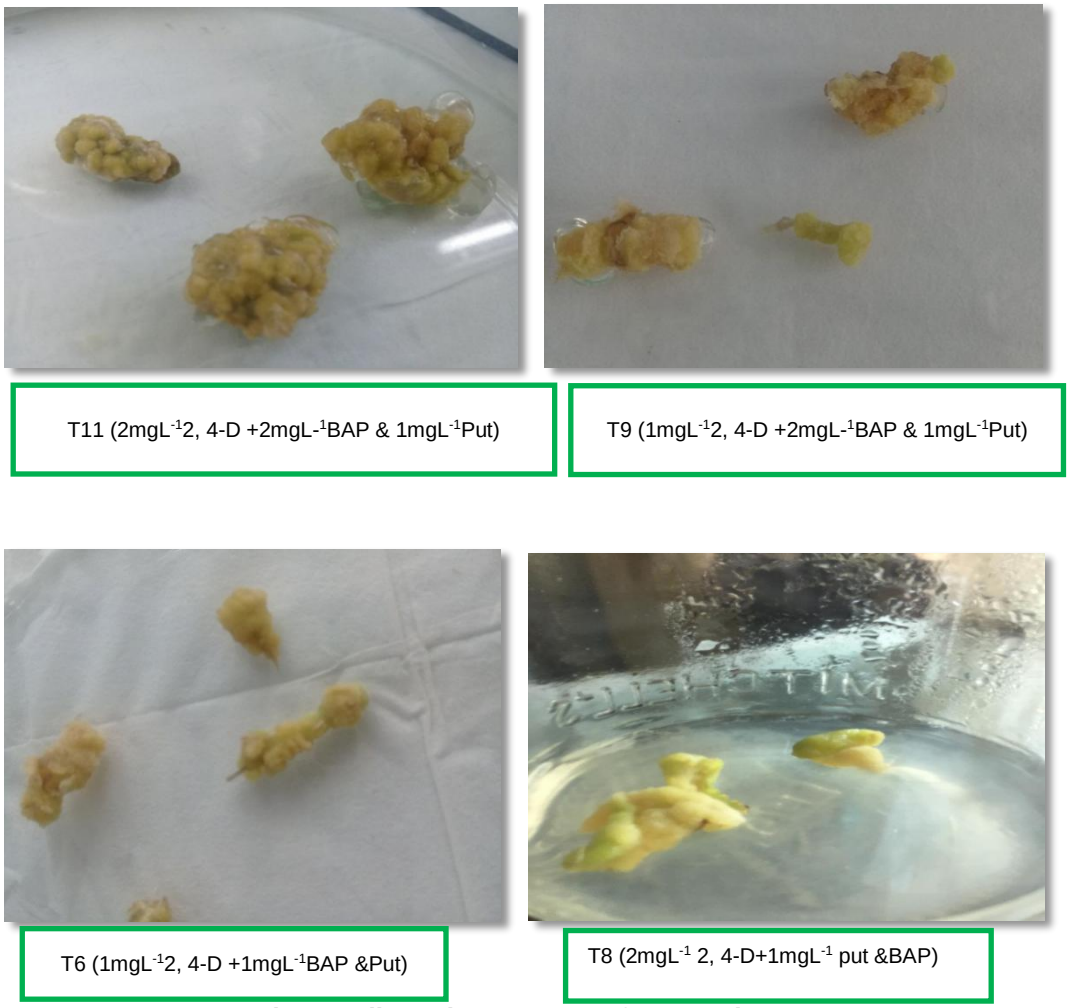


Fig 2: Callogenic response of Internode segments

CONCLUSION

Stevia rebaudiana was successfully propagated through leaf and internode segments by using successful combination of PGRs and PAs. Scientific efforts are further needed to prove medicinal potential of this plant in order to further attract botanists and tissue culturists to propagate it at a large scale considering its importance.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

This study was designed and coordinated by SAK and FMS. SI performed and prepared the manuscript. SK and SI finalized the final version.

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