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Root Induction and *Ex Vitro* Acclimatization Strategies for Efficient and Sustainable Propagation of *Stevia rebaudiana* Bertoni

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ABSTRACT

Successful establishment of micro propagated Stevia rebaudiana Bertoni depends on effective rooting and ex vitro adaptation. This study evaluated the effects of NAA and IBA on rooting and acclimatization. IBA at 1.0 mg/L produced favourable responses. BAP-derived nodal plantlets recorded 12.0 roots per shoot and 93.4% survival, while leaf-derived plantlets showed 9.3 roots and 75.2% survival. Kinetin-derived nodal plantlets recorded 10.0 roots and 91.1% survival, whereas BAP–Kn–D-biotin-derived plantlets showed 7.4 roots and 92.0% survival. Plantlets were hardened in a cocopeat–vermiculite mixture (1:1), highlighting the importance of auxin treatment and controlled acclimatization.

Keywords: *Stevia rebaudiana; micropropagation; root induction; NAA; IBA; acclimatization; ex vitro survival; plant establishment.*

Introduction

The perennial herb *Stevia rebaudiana* Bertoni, which is well-known for its medicinal properties, belongs to the Asteraceae family. It is widely farmed for its sweet diterpene glycosides, stevioside and rebaudioside-A, which are incredibly sweet without adding any calories and are about 200–300 times sweeter than sucrose[1-2]. Stevia's anti-obesity, anti-diabetic, antihypertensive, and antioxidant qualities have piqued the interest of the pharmaceutical, nutraceutical, and food industries [3]. Due to low seed viability, low germination rates, genetic variability, and restricted seed output, conventional seed propagation of Stevia rebaudiana is frequently unreliable [4]. Low rates of multiplication and seasonal fluctuations also limit vegetative propagation via stem cuttings [5]. Consequently, plant tissue culture methods have become a viable substitute for large-scale propagation, allowing for the year-round quick generation of genetically homogeneous and disease-free planting material [6].

Culture establishment, shoot multiplication, roots, and acclimation are some of the steps involved in *S. rebaudiana* micropropagation [7]. Among these, successful plant establishment is thought to depend critically on roots and ex vitro acclimation [8]. Shoots produced in vitro typically have underdeveloped root systems and physiological anomalies such diminished cuticular wax deposition, non-functioning stomata, and restricted photosynthetic potential. As a result, when plantlets are moved from controlled growth conditions to natural settings, they frequently suffer significant transplant shock [9]. The induction of a well-developed root system is essential for efficient water and nutrient uptake, thereby enhancing plant survival during acclimatization [10].

In micro-propagated plantlets, auxins such α-naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) are frequently used to promote root development [11]. Auxin type and concentration have been shown to have a major impact on rooting efficiency, root quality, and subsequent field establishment [12]. Additionally, to help plantlets gradually adjust to ex vitro conditions, suitable hardening substrates and regulated environmental conditions are required [12-13]. Although successful protocols for shoot multiplication of *S. rebaudiana* have been reported information regarding the comparative rooting responses and acclimatization success of plantlets derived from different regeneration pathways remains limited. Therefore, the present study was undertaken to evaluate the effects of NAA and IBA on root induction and to assess the acclimatization performance of micropropagated *Stevia rebaudiana* plantlets obtained from various in vitro regeneration systems. The study aims to identify optimal rooting treatments and hardening conditions that promote successful establishment and survival of micropropagated plants under ex vitro conditions.

Material and methods

Tissue culture studies

This section presents the details of material and methods which were employed for undertaking the present study.

Plant material

The mother plant was collected from the Tri Shakti farms, Sanathnagar, Hyderabad. The explants used were the Shoot Apex, Nodal Region, and Leaf Segments.

Initiation of cultures

After Surface Sterilization the explants were cut into a specific size with a scalpel on pre sterilized petri plates and embedded on to the media with various concentrations of hormones. The cultures were maintained for 4 weeks and after proper callusing they were Sub cultured on different media. All the experiments were repeated 3 times and 12 replications (Test Tubes/culture vessels) for each treatment were used each time. The culture were observed regularly to watch the growth and regeneration and recorded the data for various parameters in each experiment.

Micro propagation

Micro propagation became one of the most ways to produce crops that are difficult to propagate by conventional methods like cuttings or seeds. The application of tissue culture technique to produce virus free plants initially led to subsequent micro propagation of a great diversity of plants *in vitro.*

Days taken For the Establishment of Explants *in vitro*

Murashige and Skoog medium (1962) along with different concentrations of cytokinin BAP (0.25mg/l, 0.5mg/l, 1.0mg/l, 1.5mg/l, 2.0mg/l, 2.5mg/l) were used. The following data were collected during this experiment.

Average number of days taken for the initiation of shoot bud, first leaf, and second leaf. The mean time taken for establishment i.e., the initiation of shoot bud, first leaf, second leaf varied with different concentrations of growth regulators. The response of Initiation of Shoot bud, first leaf, second leaf was early i.e., 15 days. In the medium supplemented with different concentrations of BAP. The mean number of days taken for the emergence of first leaf varied with different concentrations of growth regulators in the basal media. The early initiation of first leaf was observed within 5 days in medium supplemented with three different concentrations of BAP.

Multiple shoots

Multiple shoots formation was observed in MS medium with different concentration of BAP. Maximum numbers of multiple shoots were obtained in media supplemented with 1.0 mg/l and significant difference was not found in low concentration of 0.5 mg/l. The mean number of days taken for the multiple shoots induction was recorded. Frequency of multiple shoots can be measured by using the formula.

$$\text{Frequency of Multiple Shoots} = \frac{\text{No. of explants that responded (by producing multiple shoots)}}{\text{Total No. of explants inoculated}} \times 100$$

1. Shoot length in cms

The shoot length of the plantlet was recorded after the 90 days of initiation and the tip of the top most leaf formed to the initiation point of roots were taken into consideration.

2. Root length in cms

The root length of the plantlet was recorded by measuring the longest root of the plantlet this was taken into records before the plantlet bottles were shifted to green house for acclimatization after 90 days of initiation.

3. Number of leaves

The total number of freshly surviving healthy green leaves both long and short were counted before the cultures bottles were shifted to green-house 90 days for further process of acclimatization the leaf at the tip of the plantlet to the lower point near the rooting area were taken into consideration.

4. Number of roots

The total number of roots per each plantlet after 90 days was taken into account before shifting them to green-house all the roots were thoroughly washed with water and roots were cleaned from medium in which it was inoculated.

$$\text{Frequency of Rooting} = \frac{\text{No. of rooted shoots}}{\text{Total No. of inoculated explants}} \times 100$$

Results

Root induction

The elongated regenerated shoots with fully expanded leaves were transferred to the rooting medium. Efficient rooting is important for successful transplantation and survival of the *in vitro* regenerated plantlets. Among different auxins tested NAA an IBA promoted the induction of roots efficiently._Shoots obtained from the *in vitro* proliferated shoots (BAP treated) were implanted on MS. Medium supplemented with different concentrations of NAA (0.25mg/l, 0.5mg/l,1.0mg/l,1.5mg/l, 2.0mg/l, 2.5mg/l) for rooting. Maximum root induction percentage was observed in medium fortified with NAA (1.5mg/l). The root induction gradually decreased with increasing concentration of Auxin. The mean number of roots per plantlets was recorded after 90 days of initiation for both the micro cuttings obtained from nodal and leaf segments. In plants generated nodal region mean number of roots /shoots (11.67) was recorded and (9.25) mean number of roots per shoots was recorded for leaf region micro cuttings (Table-1, Graph-1). The percentage of response of rooting from nodal region was about 11.7% and 9.2% in leaf region for plants BAP (1.0mg/l) (Table-2). The percentage of survival of pants in nodal region was 91.3% and in case of leaf region it was 75% (Table-2). After 90 days of initiation the length of the root was taken into consideration. Root length of about 16.5cms in nodal region and 14.0cms in leaf region was observed. Similarly, Kn+NAA in nodal explants mean number of roots /shoots (7.7) and in case of BAP+Kn+D-Biotin+NAA it was recorded (7.9) (Table -3,4; Graph-3,4). The percentage of response of rooting from nodal region (Kn) was about (8.0) and for BAP+Kn+D-Biotin (9.0). The percentage of survival of pants in nodal region (Kn) 92% and for BAP+Kn+D-Biotin 92%. Root length of about 16.1cms in nodal region regenerated plants from Kn 16.2cms of rot length was recorded in nodal region regenerated plants from BAP+Kn+D-Biotin (Table-5,6).

Preliminary experimental studies were carried out for root induction using IBA with different concentrations (0.25mg/l, 0.5mg/l, 1.0mg/l, 1.5mg/l, 2.0mg/l, 2.5mg/l) for all the shoots proliferated from all the combinations used BAP, Kn, BAP+Kn+D-Biotin. For BAP treated plants mean number of roots /shoots (12.0) for nodal region and (9.3) for leaf region was recorded (Table-7, Graph-7). The percentage of response of rooting from nodal region was about 11.7 for nodal and its 9.2 for leaf region. The percentage of survival of plants in nodal region is 93.4% and its 75.2% in case of leaf region which is similar to that of NAA concentration treatments.

IBA (1.0mg/l) showed good rooting. In (Kn) concentration in nodal region mean number of roots /shoots (10.0) was recorded (Table-3, Graph-3) . The percentage of response of rooting from nodal region was about 10.0. The percentage of survival of pants in nodal region is 91.1% (Table-1). Mean number of roots /shoots (7.4) was recorded (Table-4). The percentage of response of rooting from nodal region was about 10.3. The percentage of survival of pants in nodal region is 92.0 % for the combination BAP+Kn+D-Biotin (Table-2).

Table 1: Rooting table for invitro regenerated grown plants in (NAA-mg/l)

Growth Regulator concentration BAP(mg/l)	No. of roots/shoot in Nodal region (Mean ± SD)	No. of roots/shoot in Nodal region (Mean ± SD)
0.25	10.0 ± 0.85	10.0 ± 0.79
0.5	11.67 ± 1.67	9.25 ± 0.75
1.0	10.33 ± 2.01	8.5 ± 1.0
1.5	10.67 ± 1.67	8.25 ± 0.75
2.0	10.00 ± 1.90	7.16 ± 0.71
2.5	8.58 ± 1.44	5.83 ± 0.93

GRAPH-3:

Rootingfor (BAP) concentrated *in vitro* regenerated plants

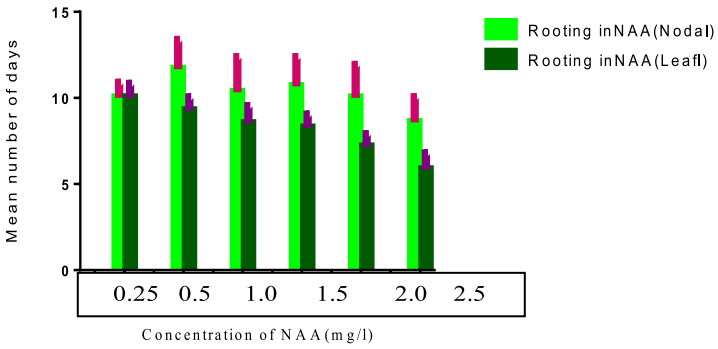


Table 2: Effect of NAA on Percentage of rooting, survival and Root length from Inter nodal and Leaf region

Growth Regulator concentration BAP (mg/l)	Percentage response of rooting (%) Nodal region	Percentage of survival (%) Nodal region	Percentage response of rooting (%) Leaf region	Length of root (cm) Nodal Region	Length of root (cm) Leaf region	Percentage of survival (%) Leaf region
0.25	10	93.2	6.1	16.4	13.9	74.5
0.5	11.7	93.4	9.2	16.5	14	75.2
1.0	10.3	91.5	8.5	16.23	13.8	75.0
1.5	10.7	91.3	8.2	16.24	13.7	75.0
2.0	10.0	91.2	7.2	16.25	13.6	74.5
2.5	8.6	91	5.9	16.24	13.5	74.3

Table 3: Rooting table for in vitro regenerated plants in Kn concentrations

Growth Regulator concentration Kn (mg/l)	No. of roots/shoot in Nodal region (Mean ± SD) IBA	No. of roots/shoot in Nodal region (Mean ± SD) NAA
0.25	7.2±1.0	5.0±0.85
0.5	10±1.1	7.7±1.1
1.0	6.8±1.2	6.9±1.0
1.5	6.7±0.98	6.3±1.1
2.0	6.1±0.79	5.0±0.85
2.5	5.5±1.4	4.7±1.2

GRAPH-2:

Rootingfor (Kn)concentrated *In vitro* regenerated plants

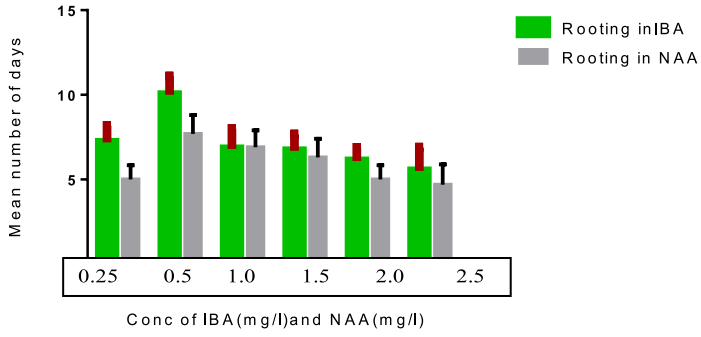


Table 4: Rooting table for in vitro regenerated plants in (BAP+Kn+D-Biotin)

Growth Regulator concentration BAP+Kn+D-Biotin (mg/l)	No. of roots/shoot in Nodal region (Mean ± SD) IBA	No. of roots/shoot in Nodal region (Mean ± SD) NAA
0.05+0.025+0.01	6.8±1.1	7.2±1.5
0.1+0.05+0.025	7.4±1.1	7.9±1.3
0.2+0.1+0.05	10.0±1.0	8.1±1.5
0.275+0.175+0.1	5.6±1.5	6.6±1.2
0.3+0.2+1.5	6.1±0.94	5.5±1.3

GRAPH-4:

Rootingfor (BAP+Kn+D-Biotin)concentrated *In vitro* regenerated plants

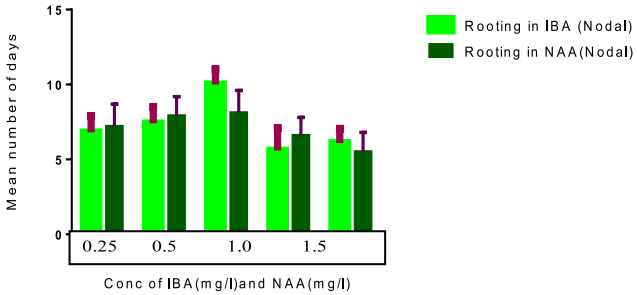


Table 5: Percentage of response of rooting, survival and root length for (Kn) in (NAA) concentration

Growth Regulator concentration Kn(mg/l)	Percentage response of rooting (%) in NAA Nodal region	Percentage of survival (%) in NAA Nodal region	Length of root (cm) in NAA Nodal region
0.25	5.0	91.1	16.0
0.5	8.0	92.0	16.1
1.0	7.0	91.0	15.8
1.5	6.2	90.3	15.7
2.0	5.0	90.0	15.6
2.5	5.0	90.0	15.5

Table 6: Percentage of response of rooting, survival and root length for (Kn) in (IBA) concentration

Growth Regulator concentration Kn(mg/l)	Percentage response of rooting (%) in IBA Nodal region	Percentage of survival (%) in IBA Nodal region	Length of root (cm) in IBA Nodal Region
0.25	7.1	91.0	16.0
0.5	10.0	91.1	16.1
1.0	7.0	91.0	15.8
1.5	7.0	90.1	15.7
2.0	6.0	90.0	15.6
2.5	5.5	89.4	15.5

Table 7: Rooting table for (BAP) concentrated plants in (IBA- 1.5mg/l)

Growth Regulator concentration BAP (mg/l)	No. of roots/shoot in Nodal region (Mean ± SD)	No. of roots/shoot in Leaf region (Mean ± SD)
0.25	10.0 ± 0.9	6.1 ± 0.8
0.5	12.0 ± 1.7	9.3 ± 0.8
1.0	9.5 ± 1.0	8.5 ± 1.0
1.5	11.0 ± 1.7	8.3 ± 0.8
2.0	10.0 ± 1.9	7.2 ± 0.7
2.5	8.6 ± 1.4	5.8 ± 0.9

GRAPH-7:

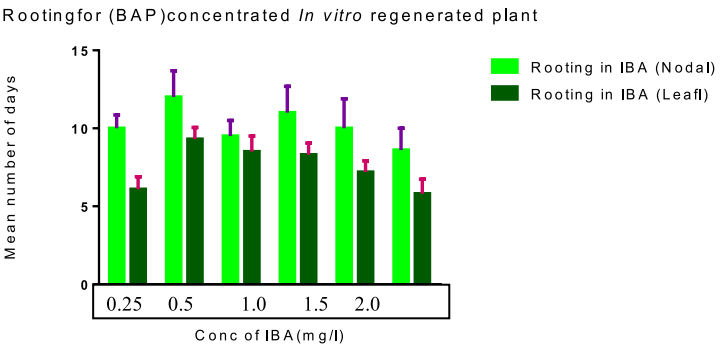


Table 8: Percentage of response of rooting, survival and root length for (Kn) in (IBA) concentration

Growth Regulator concentration Kn (mg/l)	Percentage response of rooting (%) in IBA Nodal region	Percentage of survival (%) in IBA Nodal region	Length of root (cm) in IBA Nodal Region
0.25	7.1	91.0	16.0
0.5	10.0	91.1	16.1
1.0	7.0	91.0	15.8
1.5	7.0	90.1	15.7
2.0	6.0	90.0	15.6
2.5	5.5	89.4	15.5

Table 9: Percentage of response of rooting, survival and root length for (BAP+Kn+D-Biotin) in (NAA) concentration

Growth Regulator concentration BAP+Kn+D-Biotin(mg/l)	Percentage response of rooting (%) in IBA Nodal region	Percentage of survival (%) in IBA Nodal region	Length of root (cm) in IBA Nodal Region
0.05+0.025+0.01	7.0	91.1	16.1
0.1+0.5+0.025	7.5	91.4	15.9
0.2+1.0+0.05	10.3	92.0	16.2
0.275+0.175+0.1	6.0	91.0	15.8
0.3+0.2+1.5	6.0	91.0	15.7

Hardening of plantlets

After rooting the plant is shifted to green house for hardening. Here the explants are washed with fungicide (Bavistin) neatly and then shifted to plastic cavities for primary hardening. After 14 days healthy root development has been observed. Then the whole *Stevia* plants are shifted to pots and ready for outlet. The plantlets were maintained at temperature of 30°C± 2°C and relative humidity of 80 90 %. All the experiments were repeated thrice and 12 replicates were maintained for each treatment. Statistical analysis was carried out by Skewness and kurtosis test.



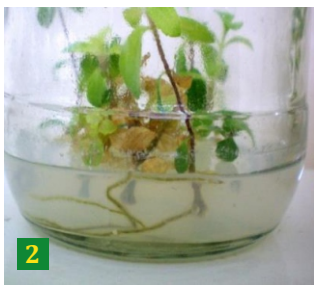










Fig: (1-6) Root formation in Micro propagated plants

Discussion

Auxin supplementation had an impact on *Stevia rebaudiana* root induction, according to the current study. Although the roots were described as hairy and thin, maximum root induction was obtained with MS media containing 1.5 mg/L of NAA. The importance of auxin supplementation is highlighted by the absence of roots in the auxin-free media. Nodal explants showed better rooting capacity than leaf explants. It also mentioned better survivability upon acclimatization. Plantlets were stored at 30 ± 2°C and 80–90% relative humidity in sterile cocopeat and vermiculite (1:1). These findings demonstrate the importance of delayed hardening and roots for a plant's successful establishment.

Conclusion

The study emphasizes the significance of roots and acclimation in *Stevia rebaudiana* Bertoni micropropagation [14-17]. The thesis found that NAA at 1.5 mg/L was the auxin treatment that was best for root induction. In terms of roots and plant survival, nodal explants outperformed leaf explants. Plantlet hardening was promoted by a two-step acclimatization process that included a 1:1 sterilized cocopeat–vermiculite mixture, high humidity of 80–90%, and a temperature of 30 ± 2°C, the results show that the successful establishment of micropropagated *Stevia rebaudiana* plants depends on appropriate auxin supplementation, efficient root development, and controlled greenhouse conditions.

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References

1. Zayed, M. S., Hegazi, G. A. E. M., Salem, H. M., & Adas, W. M. I. A. (2017). Role of endomycorrhizae and Pseudomonas uorescens on the acclimatization of micropropagated Stevia rebaudiana Bert. plantlets. *African Journal of Plant Science*, *11*(3), 38-47.

2. Zayova, E., Stancheva, I., Geneva, M., Petrova, M., & Dimitrova, L. (2013). Antioxidant activity of in vitro propagated Stevia rebaudiana Bertoni plants of different origins. *Turkish Journal of Biology*, *37*(1), 106-113.
3. Röck-Okuyucu, B., Bayraktar, M., Akgun, I. H., & Gurel, A. (2016). Plant growth regulator effects on in vitro propagation and stevioside production in Stevia rebaudiana Bertoni. *HortScience*, *51*(12), 1573-1580.
4. Humeraha Nazneen, and Priya Srivastava (2018).Comparative Biomass Accumulation in Micro propagated and Somatic Embryo-Derived Stevia rebaudiana.Life Science Review. DOI: https://doi.org/10.51470/LSR.2018.02.02.05
5. Humeraha Nazneen, Brajendra, and S. Karnakar Reddy (2017). HPLC-Based Estimation of Stevioside in In Vivo and In Vitro-Grown Stevia rebaudiana.Life Science Review. DOI: https://doi.org/10.51470/LSR.2017.01.01.09
6. Ibrahim, I. A., Nasr, M. I., Mohammedm, B. R., & El-Zefzafi, M. M. (2008). Nutrient factors affecting in vitro cultivation of Stevia rebaudiana. *Sugar tech*, *10*(3), 248-253.
7. Sreedhar, R. V., Venkatachalam, L., Thimmaraju, R., Bhagyalakshmi, N., Narayan, M. S., & Ravishankar, G. A. (2008). Direct organogenesis from leaf explants of Stevia rebaudiana and cultivation in bioreactor. *Biologia Plantarum*, *52*(2), 355-360.
8. Tomaszewska-Sowa, M., Sawilska, A. K., & Figas, A. (2015). In vitro shoots regeneration and plantlets acclimatization of Stevia rebaudiana Bertoni ssp. Sweety. *BioTechnologia. Journal of Biotechnology Computational Biology and Bionanotechnology*, *96*(1).
9. Rosales, C., Brenes, J., Salas, K., Arce-Solano, S., & Abdelnour-Esquivel, A. (2018). Micropropagación de Stevia rebaudiana en sistemas de inmersión temporal para incursionar en la producción hortícola. *Revista Chapingo. Serie horticultura*, *24*(1), 69-84.
10. Taleie, N., Hamidoghli, S., & Hamidoghli, Y. (2012). In vitro plantlet propagation of Stevia rebaudiana Bertoni. *South West. J. Hortic. Biol. Environ*, *3*(1), 99-108.
11. Rezaie, S., Dehestani-Ardakani, M., & Kamali, K. (2018). A new protocol for direct regeneration of stevia plant (Stevia rebaudiana Bertoni) by tissue culture techniques. *Journal of Horticulture and Postharvest Research*, *1*(2), 97-104.
12. Modi, A. R., Patil, G., Kumar, N., Singh, A. S., & Subhash, N. (2012). A simple and efficient in vitro mass multiplication procedure for Stevia rebaudiana Bertoni and analysis of genetic fidelity of in vitro raised plants through RAPD. *Sugar Tech*, *14*(4), 391-397.
13. Gopal Kumar, R S Kurothe, Brajendra, A K Vishwakarma, B K Rao and V C Pande (2014) Effect of farmyard manure and fertilizer application on crop yield,runoff and soil erosion and soil organic carbon under rainfed pearl millet (Pennisetum glaucum). *Indian Journal of Agricultural Sciences* 84 (7): 816–23
14. Gantait, S., Das, A., & Mandal, N. (2015). Stevia: a comprehensive review on ethnopharmacological properties and in vitro regeneration. *Sugar Tech*, *17*(2), 95-106.
15. Gupta, P., Sharma, S., & Saxena, S. (2016). Effect of abiotic stress on growth parameters and steviol glycoside content in Stevia rebaudiana (Bertoni) raised in vitro. *Journal of Applied Research on Medicinal and Aromatic Plants*, *3*(4), 160-167.
16. P. Srilatha, Faisal Yousuf, Ramesh Methre, T. Vishnukiran, Surekha Agarwal,Yugandhar Poli, M. Raghurami Reddy, B. Vidyasagar, Chitra Shanker, D. Krishnaveni,S. Triveni, Brajendra, Shelly Praveen, S.M. Balachandran, D. Subrahmanyam,Satendra K. Mangrauthia.(2019). Physical interaction of RTBV ORFI with D1 protein of Oryza sativa and Fe/Znhomeostasis play a key role in symptoms development during rice tungro disease to facilitate the insect mediated virus transmission. *Virology* 526 (2019) 117–124
17. Sharma, S., Walia, S., Singh, B., & Kumar, R. (2016). Comprehensive review on agro technologies of low-calorie natural sweetener stevia (Stevia rebaudiana Bertoni): a boon to diabetic patients. *Journal of the Science of Food and Agriculture*, *96*(6), 1867-1879.