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Research Article

In vitro regeneration of *Stevia* and evaluation of antimicrobial and antiprotozoal properties of regenerated calli and plants

Arvind Arya1*, Sandeep Kumar[2], and M.S. Kasana[3]

[1] Department of Biotechnology, Meerut Institute of Engineering and Technology, Meerut. UP, India
[2] National Institute of Engineering and Technology, NIMS University, Jaipur
[3] Department of Botany, IP College, Bulandshar, UP, India
* E-mail: arvindarya@hotmail.com

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Abstract

Stevia a 'Latin American herb' is the world's only natural sweetener with zero calories, zero carbohydrates and a zero glycemic index. In the present investigation, the *in vitro* regeneration of *Stevia rebaudiana* was performed through callogenesis and organogenesis from different explants (Leaves, Inter node, Shoot discs). Leaves explants showed best callus induction response when cultured on MS + (2.0 µM BAP + 1.0 µM NAA). However, the best callus initiation from leaf explants was obtained on MS medium supplemented with 1.0 µM NAA + 1.0 µM Kn. Optimization results of medium type and carbon source confirm 1X MS medium and glucose (3%) respectively best for callus initiation. Shoot initiation was achieved on 1X MS medium supplemented with 5.0 µM BAP + 1.0 µM NAA. *In vitro* raised shoots of *Stevia* showed best rooting on 1X MS + (1.0 µM IAA). The methanolic extracts of regenerated plants and callus culture of *Stevia* showed best antibacterial and antifungal activity against a number of microorganisms. Antiprotozoal activity of methanolic extract was also tested and found satisfactory. *In vitro* regeneration protocol of *Stevia* through callus culture is advantageous for enhanced multiplication of superior *Stevia* cultivars. Furthermore, callus from leaf explants can be a good source for production of antimicrobial and antiprotozoal compound of *Stevia* through bioreactor.

Keywords
Antibacterial, Antifungal, Antiplasmodial, Antiprotozoal, Auxin.

Introduction

Stevia 'the sweet herb of Paraguay' contains a number of diterpene steviol glycosides which are about 300 times sweeter than sucrose at their concentration of 4% (w/v) (Brandle *et al*., 1998). Besides its sweetening property *Stevia* is also known for its medicinal properties (Debnath, 2008; Ali *et al*., 2010). In India and in other countries scientists have developed micropropagation protocol for the regeneration of *Stevia* through different *in vitro* regeneration pathways viz. bud induction (Sreedhar *et al*., 2008), somatic embryogenesis (Bespalhok *et al*., 1997; Das and Mandal, 2010) and organogenesis from callus culture (Sreedhar *et al*., 2008; Patel and Shah, 2009).

Callus initiation in *Stevia rebaudiana* is reported from different explants viz. leaves shoot tip and shoots discs (Bespalhok *et al*., 1997; Banerjee and Sarkar, 2008; Das and Mandal, 2010). Fewer reports are available on the regeneration of *Stevia* from unorganized callus tissues derived from different explants by dedifferentiation induced by exogenous growth regulators (Bondarev *et al*., 2003; Patel and Shah, 2009).

Organogenesis through callus cultures facilitates the amplification of limiting plant material and the isolation of rare somaclonal variants (Rout *et al*., 2000). Another popular aspect of callus culture is the production of secondary metabolite as it cannot be synthesized economically on commercial basis (Vaniserce *et al*., 2004).

Plant materials are an important source of medicinal and pesticide components. Medicinal plants now recognized as an effective and environmental friendly material to substitute the most dangerous synthetic chemicals.

Since micropropagation of *Stevia rebaudiana* is one way to increase the biomass of this medicinal plant, together with the development and photochemical characterization of new variety of *Stevia rebaudiana* with higher level of steviol glycoside. Therefore, the primary aim of present investigation was to develop a well standardize micropropagation protocol for *Stevia* through callus culture technique and to compare the antimicrobial, antifungal and antiprotozoal potential of calli and regenerated plants.

Material and methods

Collection and sterilization of plant material: Attempts were made to induce callus from different explants (leaves, inter-nodes and shoot discs) of Stevia rebaudiana. All three explants were collected from young and old-field grown plants of Stevia at MIET, Meerut. Shoots from mature plants and young plants collected and after the initial washing with running tap water were cut into small pieces 1-2 mm thick disc and to a length of 5-6 cm from the tip for culturing. Final sterilization of different explants was performed individually using 0.1% $HgCl_2$ for 5 – 10 min and rinsed several times with autoclaved distilled water inside laminar hood.

Callogenesis: Young and old explants viz. leaves, inter-nodes and shoot discs were initially cultured on MS medium supplemented with 2.0 µM BAP + 1.0 µM NAA.

To assess the effect of auxins and cytokinins concentrations on callus induction the explants viz. leaves, inter-nodes and shoot discs were further cultured on MS medium supplemented with 2,4 – D (0.5 - 5.0 µM) or NAA (0.5- 3.0 µM) alone. BAP or Kinetin (1.0 - 5.0 µM) were also tested alone supplemented in MS medium. A combined effect of various concentrations of auxins (2,4 – D and NAA) and cytokinins (BAP and Kn) were also tested for initiation of callus from different explants of *Stevia*.

To study the effect of auxin-cytokinin interaction on shoot induction from callus cultures, 500 mg of callus were sub-cultured on MS medium supplemented with varying concentrations (0.1, 0.5, 1, 2, 3, and 4 µM) of BAP alone and (0.1, 0.2, 0.5, 1 and 2 µM) NAA in combination with 5 µM BAP . Data were recorded after four weeks of culturing.

Rooting: For rooting, the *in vitro* multiplied shoots of *Stevia rebaudiana* were transferred on rooting medium. Half strength MS medium was supplemented with different concentrations (0.2, 0.5, 1 and 2 µM) of three auxins viz. IAA, IBA, and NAA. Data were recorded after 6 weeks of culturing.

Extraction: Proliferated calli (40 g FW) and regenerated whole plant (30 g FW) were air-dried and pulverized in 100 ml of organic solvents viz. acetone, chloroform, methanol and water. Solvents were maintained at room temperature for seven days and subsequently filtered through Whatman filter paper No. 1. The residues were again dipped in alcohol for seven days. The extracts were combined and evaporated using rotary evaporator. The plant and callus extracts obtained thus were used for antibacterial, antifungal and antiprotozoal assessment.

Antibacterial activity**:** To analyze the antibacterial activity of regenerated plant and callus extracts of Stevia, different bacterial strains were used viz. Bacillus cereus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Streptococcus mutans, Streptococcus pyogenes, Streptococcus salivarius and Treponema denticola. Different bacterial strains were kind gift from microbial culture bank at MIET, Meerut. Dried extracts of regenerated plant's leaves and callus at a concentration of 20 mg/ml were tested for antibacterial activity using the agar well diffusion. Petri plates seeded with nutrient agar were prepared and wells made with 8mm cork borer. Plates were incubated at 37 °C for 24 h.

Antifungal activity: Eight fungal strains viz. Aspergillus flavus, Aspergillus fumigates, Aspergillus niger, Candida albicans, Fusarium oxysporum, Mucor mucedo, Penicillium notatum, and Trichoderma citrinoviride were used in the present study. Fungal strains were cultured on their respective medium (data not given) supplemented with 1 ml (50 mg/ml) of plant extract. The growth of fungal cultures were measured and compared with control plates.

Antiprotozoal activity:To investigate the effect of regenerated plant and callus extracts of Stevia prepared in methanol, three protozoan Balantidium coli, Entamoeba histolytica, and Giardia lamblia were used. Protozoan were collected from particular sources and provided by the Microbiology Department at MIET, Meerut. The protozoan were cultured on complex medium as mentioned in a previous report by George and Benny, 2010. Antiprotozoal test was performed by microscopic count of protozoan. Test samples were prepared by adding methanolic extracts of regenerated plants, callus extracts of Stevia (1 ml per 4 ml of protozoan culture), and 200 µl of test sample was used for microscopic count. Number of motile and non-motile organisms were counted in consequence of the antiprotozoal activity of methanolic extracts of Stevia. The test was performed in five replicates.

Results

Callogenesis: Young leaf explants of *Stevia* showed the best callus induction response (79%) and developed callus at cut surfaces, which subsequently covered the entire surface within 15-20 days. Internodes and shoot discs explants produced brownish callus, which showed poor response in further sub-culturing. Similar results were observed from different explants collected from old plants of *Stevia* (Table1).

A more detailed view was collected by subjecting different explants to varying concentrations and

combinations of cytokinins (BAP and Kn) and auxins (2,4 – D and NAA). The different explants cultured on the MS medium supplemented with different concentrations of 2,4 – D and NAA showed best callusing response on 2.0 µM 2,4 – D from leaf explants (73%) followed by inter-node (68%) and shoot discs (58%) explants of *Stevia*. NAA also showed optimum callusing at 1.0 µM concentration from all three explants of *Stevia* (Fig 1 A). From different concentrations of BAP and Kn alone the best callusing response was achieved 2.0 µM BAP (71%) and 1.0 µM Kn (78%) (Fig 1 B). 2,4 – D (2.0 µM) alone was found best for the induction of callus from different explants. However the days of callus induction were not same for different explants. Still the leaf explants showed the fast callus induction compared to other explants. From different combination, the best callusing was obtained on NAA (1.0 µM) + Kn (1.0 µM). At this concentration, the callus was whitish green and globular. Several other combinations of auxins and cytokinins also showed good callus induction response from different explants of *Stevia* (Fig 1 C). Callus obtained from different explants were subcultured and maintained on the same medium.

Organogenesis: The best organogenic response was achieved on BAP (5.0 µM) + NAA (1.0 µM) (Fig 1 D). The shoots thus produced were healthy and vigorous. However, the shoots regenerated on other concentrations of phytohormones were unhealthy and showed stunted growth.

Rooting:*In vitro* proliferated shoots were transferred to auxins containing medium for root induction. Among the auxins, the best root induction response (11.85 ± 0.45 cm) was observed in medium supplemented with IAA (1.0 µM) (Fig 1 E). With increase in the auxins concentrations, the root induction declined.

Antibacterial activity:
Rooted plantlets of *Stevia rebaudiana* were collected from cultures and extracted in four different solvents viz. Acetone, Chloroform, Methanol and Water. Similarly, proliferated callus cultures were also extracted using the four solvents and were tested for antibacterial activity on ten different strains of bacteria. Regenerated plant extracts in methanol showed maximum antibacterial response in most of bacterial strains followed by acetone and chloroform extracts (Fig2). Methanol extracts showed maximum activity against *Bacillus cereus* followed by *Pseudomonas aeruginosa* and *Staphylococcus aureus.* While four bacterial strains viz. *Streptococcus mutans, Streptococcus pyogenes, Streptococcus salivarius* and *Treponema denticola* showed almost similar response with methanol and acetone extracts of regenerated *Stevia* plants (Fig3). Chloroform extract of regenerated plants showed least significant inhibition of bacteria as compared to other two solvents. However, the extract prepared in water showed almost nil response.

Callus extract of *Stevia rebaudiana* in four different solvents showed nearly 50% less response compared to plant extract. Methanol and acetone extract showed maximum inhibition than chloroform with different strains of bacteria. Water extracts again showed nil response.

Antifungal activity
Antifungal activity of regenerated plant and callus extracts in four different solvents were tested on eight different fungal strains of which *Aspergillus flavus, Aspergillus fumigates, Aspergillus niger* and *Fusarium oxysporum* showed maximum inhibition by methanolic plant extracts of *Stevia rebaudiana* (Fig4). Similar results were obtained with callus extracts prepared in methanol and acetone. Water extracts of plants and callus of *Stevia* were found non-responsive and showed no antifungal activity of the extracts (Fig5).

Antiprotozoal activity
Antiprotozoal activity of regenerated plant and callus extracts of *Stevia* against three protozoan are shown in Table2 and Table3 respectively. These preliminary antiprotozoal studies signify success of methanolic extracts of *in vitro* regenerated *Stevia* plants and callus cultures. All three protozoans were found susceptible and showed nearly 40% drop in count.

Discussion
In the present investigation, the leaf explant responded best towards callus initiation. In a report by Huda *et al*. (2007), fast callusing response was reported from nodal explants of *Stevia* than leaf explants and contrary to this leaf explants showed much quicker response of callus initiation than other explants types in our findings. These results are in line with a previous report (Banerjee and Sarkar, 2008), where callus formation was reported from leaf, nodal segments and internodes explants of *Stevia* and also that nodal segments produce callus much faster than other explants.

Three different auxins viz. 2,4 – D, IBA and NAA were used in the present investigation at different concentrations alone and in various combinations. 2,4 – D (2.0 µM) in combination with Kn (1.0 µM) on all explants type produced callus. The combination of 2,4 – D (2.0 µM) + NAA (1.0 µM) showed the best callus induction (95%) from leaf. Role of NAA in the initiation of callus was individually studied by Huda *et al*. (2007).

Callus cultures from leaf explants were subcultured on same medium for multiplication and later transferred to cytokinins (BAP) containing medium for induction of shoots from calli. In the present research, different concentrations of BAP in association with NAA were tested and showed positive results at BAP (5.0 µM) + NAA (1.0 µM). Our findings are also in line with the previous findings (Ahmed *et al*., 2007; Ibrahim *et al*., 2008).

In the present investigation, the antimicrobial and antiprotozoal activity of regenerated *Stevia* plants and callus in four different solvent systems were studied. Antibacterial and antifungal study of *Stevia* extracts were tested against ten different bacterial strains and eight different fungal strains respectively. Results showed the positive response of methanolic and acetone extracts of regenerated plants and callus of *Stevia*. Maximum number of bacterial strains were found susceptible to *Stevia* extracts. Antibacterial property of *Stevia rebaudiana* extracts in various solvents on four bacteria viz. *E. coli, B. subtilis, S. mutans* and *S. aureus* and six different fungal strains were. However, only few fungi were found inhibited by leaf extracts by Debnath, 2008.

Water extracts of plant and callus of *Stevia* could not show any antibacterial and antifungal response. Several researchers reported the ineffectiveness of water extracts of *Stevia rebaudiana* (Tadhani and Subhash, 2006; Esmat and Ferial, 2010a; Esmat and Ferial, 2010b). However, some contrasting reports showed antimicrobial activity of hot water extract of *Stevia rebaudiana* towards *E. coli* (Tomita *et al*., 1997). Among the four solvents used in the present research, methanol and acetone were found most successful. Previous findings also showed the superiority of methanolic extract of *Stevia* leaves which was earlier reported by Esmat and Ferial, (2010b) . They reported the antibacterial activity of leaf and callus extracts of *Stevia* in six different solvents on *L. monocytogenes, S. aureus, P. aeruginosa* and *B. cereus*. Among the six extracts methanolic extracts showed greater antibacterial potential. Higher antibacterial activity of methanolic extracts is due to its greater solubility (De-Boer *et al*., 2005). Methanolic extracts are also reported to have maximum antimicrobial activity of secondary metabolites (Esmat and Ferial, 2010b). In *Stevia* the antimicrobial activity is generally attributed to "stevioside" from *Stevia* (Nakamura and Tamura, 1985). Methanolic leaf extract of *Stevia* is also reported to have maximum antioxidant activity (Shukla, 2009).

Zone of inhibition also were found to change with the concentration of plant extracts. Dilute extracts showed better zone of inhibition than pure extract probably because of the permeability and diffusivity of the dilute extract in the medium (Parekh *et al*., 2005). Methanolic extracts of *in vitro* regenerated plant and callus cultures of *Stevia* showed good sign of antiprotozoal activity against three protozoans. Several other reports also support the antiprotozoal (George and Benny, 2010; Calzada *et al*., 2005) and antiplasmodial (Simonsen *et al*., 2001) activity of *in vitro* plant extracts.

References

Ahmed, M., Salahin, M., Karim, R., Razvy, M., Hannan, M., Sultana, R., Hossain, M. and Islam, R. 2007. An efficient method for *in vitro* clonal propagation of a newly introduced sweetener plant (*Stevia rebaudiana* Bertoni.) in Bangladesh. *American-Eurasian J. Scientific Res.*,**2***:* 121–125.

Ali, P., Elmira, S. and Katayoun, J. 2010. Comparative study of the antibacterial, antifungal and antioxidant activity and total content of phenolic compounds of cell cultures and wild plants of three endemic species of Ephedra. *Molecules*, **15**: 1668–1678.

Banerjee, M. and Sarkar, P. 2008. *In vitro* callusing in *Stevia rebaudiana* Bertoni using cyanobacterial media - a novel approach to tissue culture. *Int. J. Biol.*, **3**(3): 163–168.

Bespalhok, J. C., Filho and Kazumi, H. 1997. Embryogenic callus formation and histological studies from *Stevia rebaudiana* Bert. Bertoni floret explants. *R. Bras. Fisiol. Veg.*, **9:** 185–188.

Bondarev, N. I., Sukhanova, M. A., Reshetnyak, O. V. and Nosov, A. M. 2003. Steviol Glycoside Content in Different Organs of *Stevia rebaudiana* and Its Dynamics during Ontogeny. *Biologia Plantarum*, **47**: 261–264.

Brandle, J. E., Starratt, A. N. and Gijzen, M. 1998. *Stevia rebaudiana*: its agricultural, biological, and chemical properties. *Canadian J. Plant Sci.*, **78**: 527–536.

Calzada, F., Cervantes-Martínez, J. A. and Yépez-Mulia, L. 2005. *In vitro* antiprotozoal activity from the roots of *Geranium mexicanum* and its constituents on *Entamoeba histolytica* and *Giardia lamblia*. *J. ethnopharmacol.*, **98**: 191–193.

Das, A. and Mandal, N. 2010. Enhanced development of embryogenic callus in *Stevia rebaudiana* Bert. by additive and amino acids. *Biotechnol.,* **9**: 368–372.

Debnath, M. 2008. Clonal propagation and antimicrobial activity of an endemic medicinal plant *Stevia rebaudiana*. *J. Med. Pl. Res.* ,**2**: 45–51.

De-Boer, H. J., Kool, A., Broberg, A., Mziray, W. R., Hedberg, I. and Leventors, J. J. 2005. Antifungal and antibacterial activity of some herbal remedies from Tanzania. *J. Ethnopharmacol.*, **96**: 461–469.

Esmat, A. A.-A. and Ferial, M. A.-S. 2010a. Physico-chemical assessment of natural sweeteners steviosides produced from *Stevia rebaudiana* Bertoni plant. *African J. Food Sci.*, **4**: 269–281.

Esmat, A. A.-A. and Ferial, M. A.-S. 2010b. Evaluation of bioactive compounds of *Stevia rebaudiana*

leaves and callus. *African J. Food Sci.*, **4**: 627–634.

George, S. and Benny, P. J. 2010. Antiprotozoal activity of the crude extract of *Flacourtia inermis* fruit by microscopic count method. *Int. J. Pharmaceutical & Biological Archive*, **1**: 385–388.

Huda, M. N., Ahmed, A., Mandal, C., Alam, K. A., Hossain, M. S. and Wadud, A. 2007. *In vitro* morphogenic response of different explants of Stevia (*Stevia rebaudiana* Bert.). *Int. J. Agrl. Res.*, **2**(12): 1006–1013.

Ibrahim, A., Ibrahim, M. I., Nasr, B. R., Mohammed and Mohammed, M. E.-Z. 2008. Plant growth regulators affecting *in vitro* cultivation of *Stevia rebaudiana*. *Sugar Tech.*, **10**: 254–259.

Nakamura, S. and Tamura, Y. 1985. Variation in the main glycosides of Stevia (*Stevia rebaudiana* Bertoni). *Japanese J. Tropical Agric.*, **29**: 109–116.

Parekh, J., Jadeja, D. and Chanda, S. 2005. Efficacy of aqueous and methanol extracts of some medicinal Plants for potential antibacterial activity. *Turkish J. Biol.*, **29**: 203–210.

Patel, R. M. and Shah, R. R. 2009. Regeneration of stevia plant through callus culture. *Indian J. Pharmaceutical Sci.*, **71**: 46.

Rout, G., Samantaray, S. and Das, P. 2000. *In vitro* manipulation and propagation of medicinal plants. *Biotechnology advances*, **18**: 91–120.

Shukla 2009. *In vitro* antioxidant activity and total phenolics content of ethanolic leaf extract of *Stevia rebaudiana* Bert. *Food and Chemical Toxicol.*, **47**: 2238–2343.

Simonsen, H. T., Nordskjold, J. B., Smitt, U. W., Nyman, U., Palpu, P., Joshi, P. and Varughese, G. 2001. *In vitro* screening of Indian medicinal plants for antiplasmodial activity. *J. Ethnopharmacol*., **74**: 195–204.

Sreedhar, R. V., Venkatachalam, L., Thimmaraju, R., Bhagyalakshmi, N., Narayan, M. S. and Ravishankar, G. A. 2008. Direct organogenesis from leaf explants of *Stevia rebaudiana* and cultivation in bioreactor. *Biologia Plantarum*, **52**: 355–360.

Tadhani, M. and Subhash, R. 2006. Preliminary studies on *Stevia rebaudiana* leaves: Proximal composition, Mineral Analysis and phytochemical screening. *J. Med. Sci.*, **6**(3): 321–326.

Tomita, T., Sato, N., Arai, T., Shiraishi, H., Sato, M., Takeuchi, M. and Kamio, Y. 1997. Bactericidal activity of a fermented hot water extract from *Stevia rebaudiana* Bertoni and other food-borne pathogenic bacteria. *Microbiol Immunol.*, **41**(12): 1005–1009.

Vaniserce, M., Lee, C., Nalawade, S. N., Lin, C. Y. and Tasy, H. 2004. Studies on the production of some important secondary metabolites from medicinal plants by plant tissue culture. *Botanical Bulletin of Academia Sinica, New Name: Botanical Studies*, **45**: 1–22.

Table1 Effect of different explants on induction of callus on MS + (2 µM BAP + 1.0 µM NAA). Data recorded after 4 weeks of culturing

Medium	Explant		Days of callus initiation	% callus induction	Callus texture
MS + 2 µM BAP + 1.0 µM NAA	Young	Leaf	20	79	Greenish, compact, healthy
		Inter-nodes	26	61	Greenish, compact
		Shoot discs	22	57	Brownish
	Old	Leaf	29	65	Greenish
		Inter-nodes	35	54	Brownish
		Shoot discs	29	48	Brownish

Note: (20 explants were used for each treatment)

Table2: Antiprotozoal activity of the *Stevia rebaudiana* regenerated plant's leaf extracts

Protozoan	Count (No.)	Observation of protozoa after 2 min for sensitivity / resistance	
		No. of resistant organism	No. of sensitive organisms
Balantidium coli	6±1	0	4±1
Entamoeba histolytica	7±2	0	4±1
Giardia lamblia	7±2	0	5±1

Table3: Antiprotozoal activity of the *Stevia rebaudiana* callus extracts

Protozoan	Count (No.)	Observation of protozoa after 2 min for sensitivity / resistance	
		No. of resistant organism	No. of sensitive organisms
Balantidium coli	7±1	1	3±1
Entamoeba histolytica	8±2	1	4±1
Giardia lamblia	8±2	2	4±1

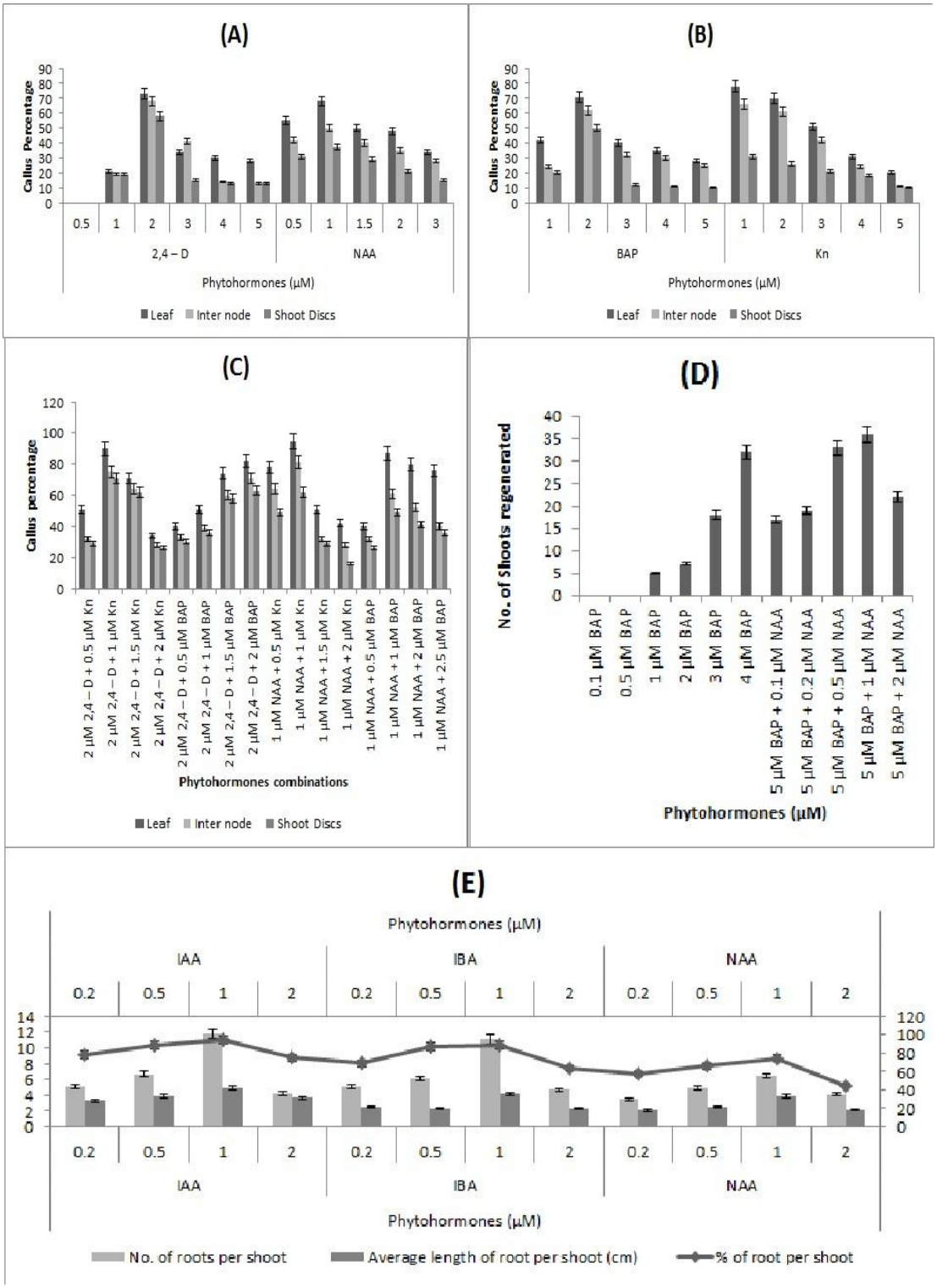

Fig 1. (A): Effect of 2,4-D and NAA on callus percentage from different explants; (B): Effect of BAP and Kn on callus percentage from different explants; (C): Effect of different combinations of phytohormones on callus percentage from different explants; (D): Effect of different phytohormones alone and in combination on regeneration of shoots; (E): Effect of auxins on rooting of in vitro raised shoots.

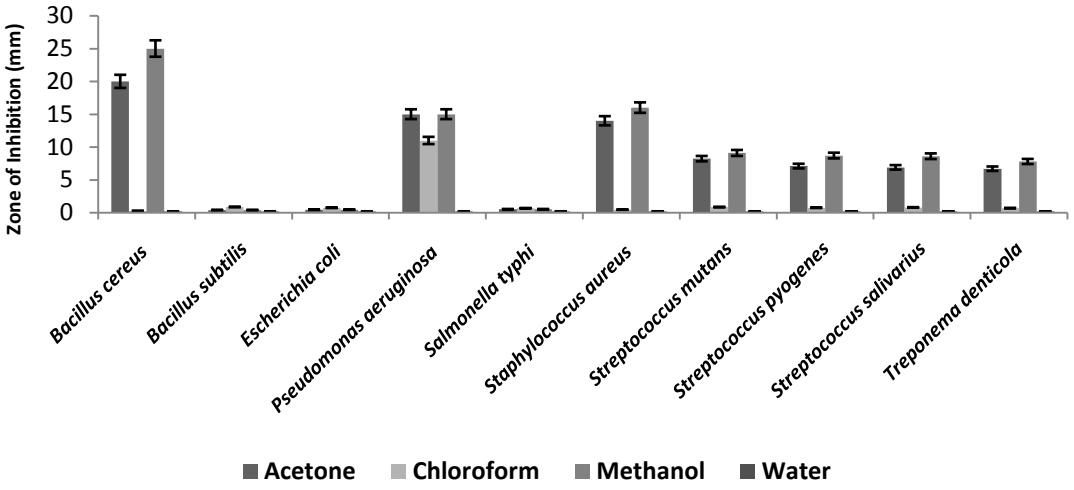

Fig2.Antibacterial activity of the *Stevia rebaudiana* regenerated plant's leaves extracts

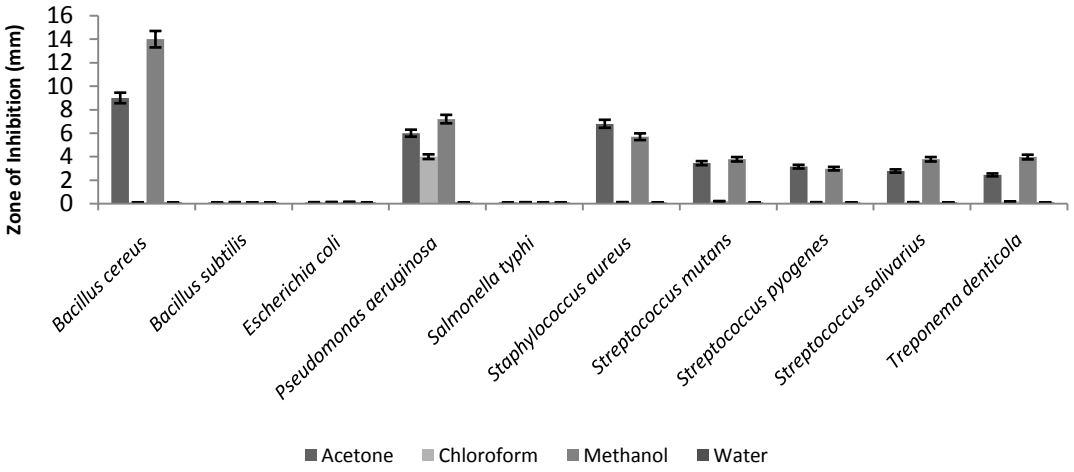

Fig3. Antibacterial activity of the *Stevia rebaudiana* callus extracts

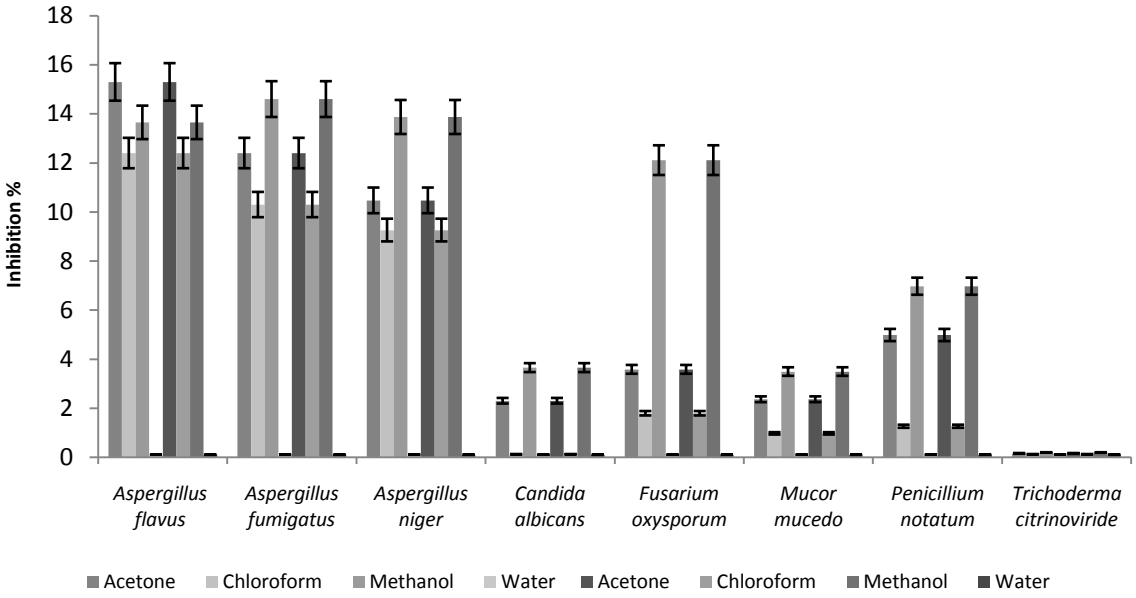

Fig4. Antifungal activity of the *Stevia rebaudiana* regenerated plants leaves extracts

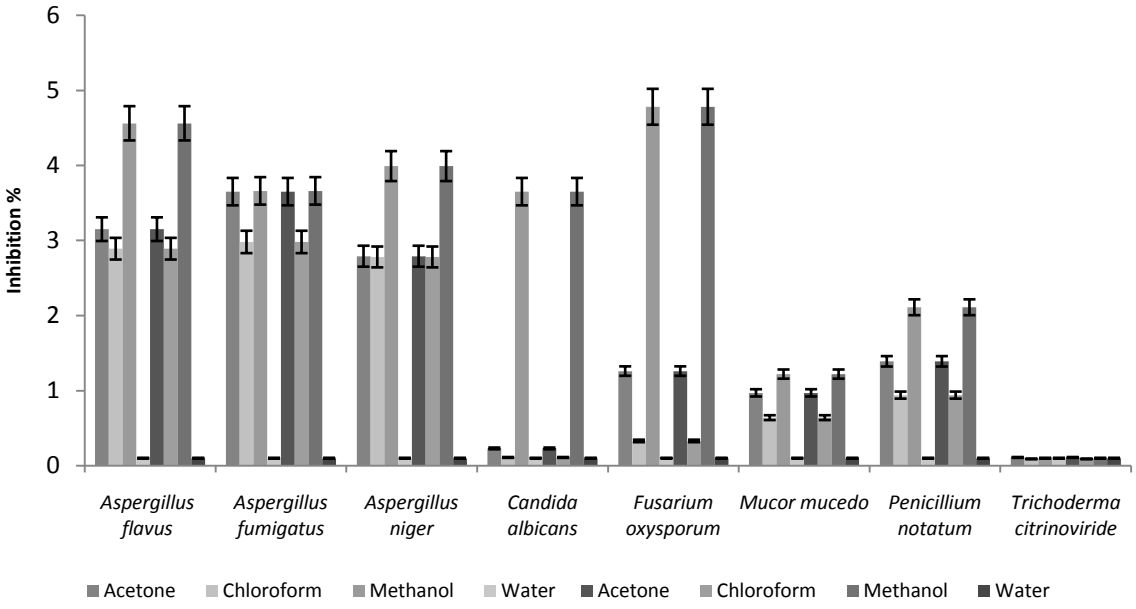

Fig5. Antifungal activity of the *Stevia rebaudiana* callus extracts