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In vitro Studies and Antimicrobial Activities of *Pereskia grandifolia* Haworth var. *grandifolia*

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ABSTRACT

In vitro propagation was developed for the medicinal plant *Pereskia grandifolia* Haworth var. *grandifolia* using axillary bud explants. Optimum multiplication of shoots was achieved on MS (Murashige and Skoog, 1962) medium supplemented with 3.0 mg/l BAP (6-Benzylaminopurine) and 30.0 mg/l adenine. Plants were maintained *in vitro* on MS medium while callus were induced on MS basal medium supplemented with the combination of 5.0 mg/l BAP (6-Benzylaminopurine) and 5.0 mg/l NAA (α-Naphthalene acetic acid). The antimicrobial tests were carried out using the crude extracts of this species from *in vitro* callus and intact leaves. No difference was detected between crude methanol extract from *Pereskia grandifolia* leaves and callus. The same extract failed to inhibit all gram positive and gram negative bacteria growth; however, it inhibited the growth of fungi. Only dermatophytes fungi (without spore) such as *Microsporium canis* and *Trichophyton rubrum* was inhibited with a medium and weak strength of methanol extract. This extract did not inhibit the growth of dermatophytes fungi (with spore) such as *Aspergillus niger* and *Trichophyton mentagrophyte*.
Keywords: *Pereskia grandifolia*, *Microsporium canis*, *Trichophyton rubru*, adenine, BAP, NAA, callus.

INTRODUCTION

Pereskia grandifolia Haworth var. *grandifolia* is a neotropical tree species which belongs to the family Cactaceae, but it differs from the typical succulent Cactaceae in its woody habit and the presence of normal leaves (Leuenberger, 1986). *Pereskia* is generally considered to be the most primitive genus within the Cactaceae. They are woody and leafy trees, shrubs and vines which do not resemble any cactus. However, since they bear areoles from which spines, branches, and leaves arise, they are true cacti. Even though *Pereskia* is a jungle cactus, they do posses characteristics which readily identify them as members of the cactus family (Ladislaus, 1956). All *Pereskia* bear large leaves which persist on the plant all year round except those that become deciduous in the dormant season. No other cactus tribe can boast this feature. The foliage of *Pereskia* is mostly non succulent, deciduous or permanent. The flowers are stalked and often growth in clusters (Leuenberger, 1986; Ladislaus, 1956). The name is also variously spelled *Peirescia*, *Peireskia*, *Perescia* and *Pereskia* (Britton & Rose, 1963). *Pereskia grandifolia* Haworth var. *grandifolia* is widespread in cultivation as an ornamental, both in and outside of Brazil and appears to be established in several countries of tropical America. Numerous collections are without indication of its native, spontaneous, or cultivated status (Leuenberger, 1986).

Members of Cactaceae can withstand high salinity and temperature in addition to their importance as ornamental plants. Bhau and Wakhlu(2001), Johnson and Emino, (1979a) and Minocha and Mehra, (1974) observed that callus of *Neomammilaria prolifera* was resistant to high concentration of plant growth regulators, which are otherwise highly toxic to higher plants. Maximum callus proliferation was stimulated in *Mammilaria elongata* in response to 2-10 mg/l 2, 4-D with complimentary levels of 1-2 mg/l kinetin or 2-ip. The callus produced was loose, friable and proliferated quickly upon transfer. Moderate amounts of friable callus were also produced in response to complimentary growth regulators, 1 mg/l IAA and 10 mg/l 2-ip (Johnson and Emino, 1979b). Primary explants of *Ferocactus acanthodes* exhibited callus growth at the cut surfaces and axillary bud growth at most or all areoles after 4-6 weeks in culture (Ault and Blackmon, 1987).

The present study was focused on tissue culture and callus production of *Pereskia grandifolia* in an attempt to study the ability of this species to produce the antimicrobial effects by using crude extracts of *Pereskia grandifolia* from *in vitro* callus cultures and intact leaves.

MATERIALS AND METHODS

Media and explants preparations for shoot multiplication and callus induction

Explants were taken from 10-12 month-old *in vitro* grown seedlings of *Pereskia grandifolia* Haworth var. *grandifolia*, which have been maintained on MS basal medium for further growth of explants. The axillary bud explants were cultured on MS medium consisted of 0.1-10.0 mg/l BAP (6-Benzylaminopurine) and 30.0 mg/l adenine. The axillary bud was sliced and cultured on medium supplemented with BAP and NAA combinations, for callus induction. MS basal medium (Murashige and Skoog, 1962) was used for this experiment. The constituent of the media was adjusted to 1 liter after the sucrose addition and the pH was adjusted to 5.7 prior to the adding of 7.0 g agar (Agar No. 3) and 0.1 g charcoal to the media. BAP (0.1 – 10 mg/l) was added later. Media was autoclaved at 121º C and 1.3 or 1.5 kPa for 15 to 20 minutes. Media were

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then dispensed into sterile plastic vials containing 20-25 ml of aliquots, inside the laminar air flow cabinet. Callus was induced on MS basal medium supplemented with the combination of 1.5-10.0 mg/l BAP and 1.5-10.0 mg/l NAA (α-Naphthalene acetic acid).

Culture Conditions

The aseptic seedlings were maintained inside the growth chamber environment, under continuous cool-white fluorescent light (50 µmol $m^{-2}s^{-1}$) at 29 ± 1º C. All explants and callus cultures were maintained in the culture room under continuous cool-white fluorescent light at 25 ± 2º C with 16 hours photoperiod and 8 hours dark.

Assessment of Culture growth

The responses of the explants were observed every week. The percentage of responded explants, number of shoots and/ or roots regenerated per explant and the average height of shoots and lengths of roots were recorded after 8 weeks in culture.

Data analysis

Data collected were statistically analyzed using analysis of variance and means were compared using Duncan's multiple range test at P=0.05.

Antimicrobial Screening

The antimicrobial screening battery consisted of: gram positive bacterium, *Bacillus subtilis*, *Staphylococcus aureus*; gram negative bacteria, *Escherichia coli, Proteus mirabilis*, *Proteus vulgaris, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*; and Fungi, *Candida albicans, Microsporium canis, Trichophyton rubrum, Trichophyton mentagrophyte and Aspergillus niger*. The antimicrobials tests were done using standard microbial test culture.

Preparation of Extracts

Fresh leaves (300 g) were dried in oven (30-35 ºC) for about 5-7 days or until sufficiently dried before extraction was carried out. Dried leaves were crushed and ground using mortar and pestle in the laboratory. The final weights of the dried powdered materials were 35 g. The leaf powder was extracted with methanol as a solvent. Extracts were filtered and concentrated to dryness using a rotary evaporator. Extract was then ready for the antimicrobial test. The same procedure was done with aseptic callus to obtain the extract of callus.

Media for Microbial Cultivation and Maintenance

Meuller Hinton Agar, Meuller Hinton Broth, Sabouraud Dextrose Agar (Difco) and Sabouraud Dextrose Broth (Difco) culture media were used. Each medium was prepared to manufacturers' specification and adjusted to the appropriate pH before sterilised by autoclaving at 121º C for 15 minutes. About 20 ml sterile agar media were poured into petri dishes and let to solidify at a slanted position in "Universal" bottles. Broth or liquid media were distributed into final containers before autoclaving.

Inoculums for Antimicrobial Testing

Cultures of fungi and bacteria grown in Mueller Hinton Broth and Sabouraud Dextrose Broth for 18 hours, respectively, were standardized to an optical density of 1 at 600 nm (OD_{600} = 1) using NOVASPEC II Visible Spectrophotometer. The density was adjusted by adding sterile broth to the cultures. The concentrations of the resultant suspensions of fungi, bacteria and yeasts were approximately 10^8 cells/ml and 10^7 cells/ml, respectively (Rahalison *et al*., 1991). The fungi and bacteria suspensions were prepared immediately before carrying out the antimicrobial assay.

Conidial suspensions of the test fungi were prepared by pouring 20 ml broth containing 1 drop Tween 80 (Sigma P-8074), into 3-day-old cultures of *Aspergillus* species or 2-week-old culture of *Trichophyton mentagrophytes*. The cultures of *Aspergillus* species and *Trichophyton mentagrophytes* were grown on Sabouraud Dextrose Agar slants at 37ºC and 27ºC, respectively. After homogenizing with glass beads, the concentrations of the resultant conidial suspensions of *Aspergillus* species and *Trichophyton mentagrophytes* were adjusted to 10^6 conidia/ml and 10^8 conidia/ml, respectively by using haemocytometer.

Semi-quantitative Antimicrobial Activity Test

According to Paper-disk Diffusion Assay method (Bauer et al., 1966; Brown and Blowers, 1978), the suspension cultures of fungi and bacteria were diluted to the final concentrations of approximately 10^6 cells/ml and 10^5 cells/ml, respectively. The bacterial suspensions were evenly spread on the surface of 4 mm thickness of Meuller Hinton Agar (MHA) plates and the fungi suspensions on Dextrose Sabouraud Agar (DSA) plates. Sterile cotton swabs were used to produce uniform growth of organisms.

Methanol (MeOH) extracts of the leaves (*in vivo*) and callus (*in vitro*) of *Pereskia grandifolia* were used as test extracts. These extracts were dissolved in methanol and applied to filter paper disks (Whatman No. 1, 6 mm in diameter), at the concentrations of 100, 200, 400 and 800 mg/ml for fungi, yeasts and antibacterial screening. After evaporation of the solvent, the disks were placed in a good contact on the seeded agar plates.

Chloramphenicol and 5-fluorocytosine (SIGMA F-7129) at the concentrations of 1.0 mg/ml were used as positive controls for antifungal and antibacterial, respectively. Saturated filter paper disks of methanol (MeOH) and blank disks were used as negative controls.

Incubation of bacteria and fungi was done at 37ºC for 24 hours. Each extract and control was employed in

triplicate for each organism. Diameters of clear zones produced around the disks (if present) were measured after the incubation time.

Agar Diffusion Assay

Following Arnone *et al*. (1994) and Drouhet *et al*. (1986), conidial suspensions of fungi test were diluted 10 times with molten Sabouraud Dextrose Agar at 40ºC and 20 ml was poured into each petri dish. Filter paper disks (Whatman No. 1, 6 mm in diameter) were impregnated with the test extract solutions in methanol (MeoH) at the concentrations of 100, 200, 400 and 800 mg/ml. The disks were transferred onto the surface of solidified agar after evaporation of solvent.

5-Fluorocytosine at the concentration of 1.0 mg/ml, saturated filter paper disks of methanol (MeoH) and blank disks were applied as controls. Three series of determination were run for each extract and species of fungi. Zones of growth inhibition were measured after 3 days incubation at 37ºC for *Aspergillus niger,* whereas a week at room temperature (27ºC) for *Trichophyton mentagrophyte and Trichophyton rubrum*.

RESULTS

The best explant source for multiple shoot formation for tested species was the axillary buds. MS medium supplemented with BAP at 1.0-10.0 mg/l was used to induce the multiple shoots. The number of shoots produced was the lowest for explants cultured in basic medium without growth regulators. Number of shoots increased with increasing concentrations of BAP. Treatment with adenine sulphate (30.0 mg/l) was the best treatment to produce healthy and luxurious leaves as compared with MS without adenine or MS supplemented with other concentrations of adenine. MS basal medium supplemented with 6.0 and 7.0 mg/l BAP and 30.0 mg/l adenine gave the highest number of shoots (4.25 ± 0.19) compared with other media such as MS supplemented with BAP alone (Table 1).

Axillary bud explants were also the best explant for producing callus, based on the weight and morphology of the callus. BAP and NAA at 5.0 mg/l were the best combinations of growth regulators for optimum and healthy callus growth. The combinations of BAP and NAA at 5.0 mg/l were used for long term culture which produced the callus weight of 2.127 ± 0.103 g and without browning of callus margin after 10 to 12 weeks in culture compared with other combinations of BAP and NAA at 6.0 - 10.0 mg/l, although higher callus weight were produced (Table 2).

Crude methanol (MeOH) extract of leaves (*in vivo*) and callus (*in vitro*) from *Pereskia grandifolia* were screened for antimicrobial activity against some human pathogenic microorganisms, which represent different existing groups of bacteria, yeast and dermatophytesDisk Diffusion Assay was used to screen the plant extracts against bacteria, yeast and dermatophytes.The Agar Diffusion Assay was employed for antifungal testing against sporulated fungi. .

Table (1): Response of axillary bud explants cultured on MS medium supplemented with BAP (0-10.0 mg/l) and adenine sulphate (30.0 mg/l) and maintained at 16 hours light and 8 hours dark at 25 ± 2ºC.

BAP concentration MS medium (mg/l)	Observations	No. of shoots (SE)	Final weight (g) (SE)	Callus size (cm) (SE)	Explants height (cm) (SE)	No. of leaves (SE)	No. of spines (SE)	No. of roots (SE)
0	Green and healthy with roots	1.00 ± 0.00	0.48 ± 0.02	0.00 ± 0.00	3.84 ± 0.10	5.30 ± 0.29	9.00 ± 0.18	3.20 ± 0.30
1.0	Green and healthy with less roots	1.00 ± 0.00	0.47 ± 0.03	0.73 ± 0.08	3.08 ± 0.09	7.05 ± 0.35	4.50 ± 0.12	0.20 ± 0.09
2.0	Green and healthy, with callus and no roots	1.10 ± 0.07	0.87 ± 0.01	1.09 ± 0.03	2.95 ± 0.10	7.10 ± 0.27	2.50 ± 0.12	0.00 ± 0.00
3.0	Green and healthy, with callus and no roots	2.50 ± 0.14	1.43 ± 0.03	1.42 ± 0.07	2.51 ± 0.07	14.80 ± 0.60	1.65 ± 0.11	0.00 ± 0.00
4.0	Green and healthy, with callus and no roots	3.55 ± 0.14	2.02 ± 0.07	1.75 ± 0.07	2.40 ± 0.08	18.90 ± 0.58	2.20 ± 0.09	0.00 ± 0.00
5.0	Green and healthy, with callus and no roots	3.10 ± 0.14	1.64 ± 0.05	1.75 ± 0.06	1.72 ± 0.07	16.20 ± 0.99	1.40 ± 0.11	0.00 ± 0.00
6.0	Green and healthy, with callus and no roots	4.25 ± 0.19	1.72 ± 0.07	1.72 ± 0.06	2.05 ± 0.07	23.10 ± 0.63	0.90 ± 0.07	0.00 ± 0.00
7.0	Green and stunted with callus and multiple shoot buds	4.25 ± 0.19	2.28 ± 0.07	1.93 ± 0.08	1.77 ± 0.06	20.40 ± 0.85	0.55 ± 0.11	0.00 ± 0.00
8.0	Greenish purple and stunted with callus and multiple shoot buds	3.50 ± 0.14	1.65 ± 0.06	1.72 ± 0.07	2.01 ± 0.08	23.20 ± 0.92	0.25 ± 0.10	0.00 ± 0.00
9.0	Greenish purple and stunted with callus and multiple shoot buds	3.35 ± 0.24	1.52 ± 0.03	1.62 ± 0.10	1.62 ± 0.06	16.15 ± 0.42	1.15 ± 0.08	0.00 ± 0.00
10.0	Greenish purple and stunted with callus and multiple shoot buds	3.20 ± 0.14	0.64 ± 0.03	1.23 ± 0.07	1.83 ± 0.09	13.45 ± 0.66	0.75 ± 0.10	0.00 ± 0.00

In each column the mean values are significantly different at 5% level.

Table (2): Response of axillary bud segments cultured in the presence of BAP (1.5-10.0 mg/l) and NAA (1.5-10.0 mg/l) and maintained at 16 hours light and 8 hours dark at 25 ± 2ºC.

BAP & NAA conc. (mg/l)	Observations	Callus fresh weight (g) (SE)	Callus dry weight (g) (SE)
1.5 BAP and NAA	Yellowish green callus proliferated around the segments	0.204 ± 0.012	0.006 ± 0.000
2.0 BAP and NAA	Yellowish green callus proliferated around the segments, no browning of callus margin	0.415 ± 0.229	0.014 ± 0.008
3.0 BAP and NAA	Yellowish green callus proliferated and cover the explant, no browning of callus margin	1.025 ± 0.016	0.034 ± 0.000
4.0 BAP and NAA	Yellowish green callus proliferated and cover the explant, no browning of callus margin	1.739 ± 0.094	0.057 ± 0.003
5.0 BAP and NAA	Yellowish green callus proliferated and cover the explant, no browning of callus margin	2.127 ± 0.103	0.070 ± 0.003
6.0 BAP and NAA	Yellowish green callus proliferated and cover the segments, browning of callus margin	2.693 ± 0.089	0.088 ± 0.003
7.0 BAP and NAA	Yellowish green callus proliferated and cover the segments, browning of callus margin	3.189 ± 0.102	0.105 ± 0.003
8.0 BAP and NAA	Yellowish green callus proliferated and cover the segments, browning of callus margin	3.393 ± 0.172	0.112 ± 0.006
9.0 BAP and NAA	Yellowish green callus proliferated and cover the explant, browning of callus margin	2.541 ± 0.184	0.084 ± 0.006
10.0 BAP and NAA	Yellowish green callus proliferated and cover the explant, browning of callus margin	2.185 ± 0.125	0.072 ± 0.004

In each column the mean values are significantly different at 5% level.

Crude methanol extract from *Pereskia grandifolia* callus failed to inhibit all bacteria growth in these experiments. *Pereskia grandifolia* callus crude methanol extract only inhibited the growth of fungi. Only dermatophytes fungi without spore such as *Microsporium canis* and *Trichophyton rubrum* can be inhibited with a medium and weak strength. *Pereskia grandifolia* callus crude methanol extract did not inhibit the growth of dermatophytes fungi with spore such as *Aspergillus niger* and *Trichophyton mentagrophyte*. For future experiments it is suggested that the water extracts and pure compounds of *Pereskia grandifolia* plant be tested on a wider range of dermatophytes fungi without spores.

Discussion

The addition of various concentrations of BAP to MS medium not only enhanced the multiplication rate but also favoured the proliferation of healthier shoots. BAP proved to be more useful compared with other cytokinins tested in shoot bud induction. According to Smith *et al*., (1991), BAP proved to be more potent cytokinin for organogenesis. Low or no auxins but moderate to high cytokinin concentrations were required for axillary shoot production (Clayton *et al*., 1990).

There was significant difference in the response of different explants types for callus formation. From the conducted experiments, the best explant for producing callus was axillary bud explants, based on the increase of callus weight at the end of the experiment. Callus proliferation was stimulated by all auxins and cytokinins tested. BAP and NAA at 5.0 mg/l each were the best combinations of growth regulators for optimum and healthy callus production. Formation of callus was observed in medium supplemented with BAP and NAA (Samantaray *et al*., 1995). The callus obtained was loose; friable and proliferated quickly upon transfer. The optimum time between subcultures to fresh medium was 8-10 weeks. This finding was similar with those found by Ault and Blackmon, (1987). Combinations of growth regulators which cause callusing also lead to greatly increased growth rates of the tissues (Minocha and Mehra, 1974).

Crude methanol extract from *Pereskia grandifolia* leaves and callus showed no difference in term of produced results, both extracts either from leaves or callus have similar activity against the microbial tested. Crude methanol extract from *Pereskia grandifolia* leaves and callus did not inhibit bacterial growth in the present conducted experiments, either gram positive or gram negative bacteria.

Pereskia grandifolia leaves and callus crude methanol extract inhibited only the growth of fungi. Only dermatophytes fungi (without spore) such as Microsporium canis and Trichophyton rubrum can be inhibited with a medium and weak strength. Pereskia grandifolia leaves and callus crude methanol extract could not inhibit the growth of dermatophytes fungi (with spore) such as Aspergillus niger and Trichophyton mentagrophyte.

We conclude that hormone BAP was the best cytokinin for shoot bud induction of this species. Axillary bud explants was the best explant source for callus formation based on weight produced. MS medium supplemented with 5.0 mg/l BAP and NAA was the optimum medium for healthy callus growth. Extracts from leaves and callus had similar effects on microbial tested. Leaves and callus extract inhibited only the growth of fungi without spores but could not inhibit the growth of fungi with spores such as *A. niger* and *T. mentagrophyte*.

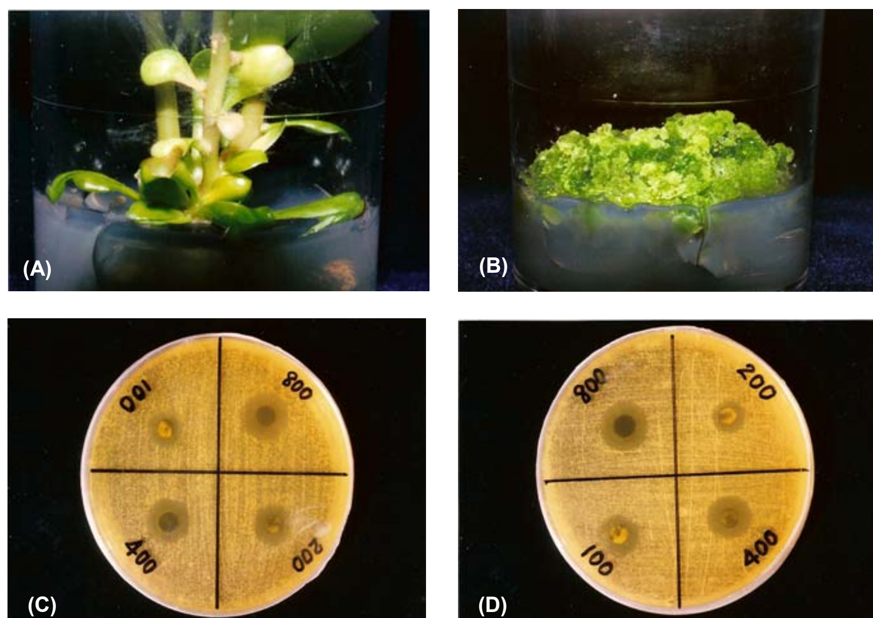

Plate (1): **(A)** Shoot formation from axillary bud explants cultured on MS medium supplemented with 3.0 mg/l BAP and 30.0 mg/l adenine sulphate, **(B)** Green callus obtained on MS medium supplemented with combinations of BAP and NAA at 5.0 mg/l, **(C)** A plate showing the inhibition zone of *Microsporium canis* (ATCC 36299) at 100, 200, 400 and 800 mg/ml extract, and **(D)** A plate showing the inhibition zone of *Trichophyton rubrum* (ATCC 28188) at 100, 200, 400 and 800 mg/ml extract.

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