Int. J. Adv. Res. Sci. Technol. Volume 6, Issue2, 2017, pp. 724-727.

IJARST

International Journal of Advanced Research in Science and Technology

journal homepage: www.ijarst.com

ISSN 2319 – 1783 (Print)

ISSN 2320 – 1126 (Online)

Studies on callus cultures of *Sterculia urens* Roxb. and estimation of secondary metabolites.

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ARTICLEINFO

Article history:

Received : 22 July 2017

Accepted : 22 Aug. 2017

Available online : 30 Aug. 2017

Keywords:

Sterculia urens,
callus cultures,
plant growth regulators,
phytochemicals.

ABSTRACT

Callus cultures were initiated from leaf explants of *Sterculia urens* on MS (Murashige and Skoog, 1962) basal medium supplemented with 0.2mg/lit TDZ and also on MS+0.5BAP+2NAA after 3 weeks. These callus cultures were used to study phytoactive molecules. Preliminary phytochemical investigation of the callus extract showed the presence of the major secondary metabolites like phenolics, tannins, flavonoids, alkaloids, carbohydrates. Quantative analysis reveals that phenolic content of (132.67±4.81), tannins (44.00±4.16), flavonoids (67.33±3.18), alkaloid (35.00±4.36) and carbohydrate content (66.00±6.43) respectively.

PAPER-QR CODE

IJARST Volume 6, Issue 2

Citation: *B. Satyanarayana. et. al. Studies on callus cultures of Sterculia urens Roxb. and estimation of secondary metabolites. Int. J. Adv. Res. Sci. Technol. Volume 6, Issue 2, 2017, pp.724-727.*

Introduction:

Sterculia urens is a moderate size deciduous tree, up to 15m height. The tree usually occurs in dry and rocky forests. The tree yields gum, known as gum karaya which has gained considerable commercial and industrial importance. The gum is also sometimes known as *Indian tragacanth*, as it is generally resembles gum tragacanth, obtained from Astragalus species (Anonymous, 1976). Gum karaya is almost entirely produced in India. The gum exudes naturally from the bark throughout the year, but most of it is generally produced by tapping or blazing by stripping off the bark.

Plant cells possess well-developed systems to regulate the level of reactive oxygen species (ROS). The overproduction of ROS in the cell is defined as an oxidative stress. They have a detrimental effect on metabolism, growth and development of cells through their ability to initiate reaction cascades that result in the production of toxic chemical species, such as hydroxyl radicals and lipid peroxides ending in cell dysfunction and death (Bowler *et al*., 1992). To regulate the level of ROS plant cells developed a complex system of antioxidants (Alscher *et al*., 1997).

Under normal tissue culture conditions many plant species fail to respond to culture manipulations (Benson, 2000a). This phenomenon, referred to as *in vitro* recalcitrance has been ascribed to several factors including cellular incompetence and necrosis etc. The three common problems for the inability of plant tissues to respond to culture manipulations for the desired outcome (Benson, 2000a) are (1) browning or necrosis of transformed cells or tissues, (2) shoot escapes in transgenesis, and (3) *in vitro* recalcitrance, these conditions severely limit the number of transgenic plants that can be regenerated. Although seemingly unrelated, recent research is beginning to unravel a common factor underlying these problems, the production of ROS, which can cause growth inhibition, cell death or alter plant metabolic pathways leading to poor regeneration of plants and the production of shoot escapes. The production of the free radicals might lead to an increase in lipid peroxidation and subsequently have a negative effect on the morphogenetic capacity of plant tissue cultures (Benson, 2000b). Antioxidant stimulated improvement in growth and regeneration further supports the relationship between oxidative stress and recalcitrance (Earnshaw and Johnson, 1987; Joy *et al*., 1988; Creemers – Molenaar and Van Oort, 1990). Plants possess various mechanisms in order to

be protected from reduced oxygen species. Protection against oxidative stress is complex and includes both enzymatic and non-enzymatic components.

Plant phenolics include a great diversity of compounds such as simple phenolics, coumarins, flavonoids, tannins, and lignin. These compounds are considered to be secondary metabolism products of plants. Higher levels of free phenolic acids in callus may participate in endogenous regulation of embryo dormancy and its further development by modification of indole-3- acetic acid metabolism (Cvikrova *et al*., 1998). High phenol content during the initial stages of culture could be associated with browning, which ultimately has an impact on tissue survival (Dalal *et al*., 1992). One of the most studied parameters in callus composition was the protein content (Blanco *et al.*, 1997, 1998). Reports of Meratan *et al* (2009) on *Acanthophyllum sordidum* shows low protein content in callus and high in regenerated shoot.

The objective of the present study is to know the phytoconstituents present in the callus cultures. This study provides information for further research work in the expression of many secondary metabolic path ways which can be easily altered by manipulating physical and chemical factors by optimizing culture conditions for further application.

Materials and methods:

Plant material:

Fresh young leaves were collected; surface sterilization was done by washing leaves with running tap water and with 5% (w/v) Teepol for 5 minutes followed by treatment with Bavistin (1%) a commercial fungicide for 5 minutes. Then the leaves were subsequently surface sterilized with 0.1% (w/v) $HgCl_2$ for 5minutes and thoroughly washed with sterile distilled water. Surface sterilized leaves were dried between Whatman No.1 filter papers and were inoculated on MS (Murashige and Skoog, 1962) basal medium and also on MS medium supplemented with Thidiazuron (TDZ, 0.1 and 0.2 mg/lit) and Benzylaminopurine (BAP,0.5 mg/lit) + Naphthalene acetic acid (NAA, 2 mg/lit). Subculturing was done after 21 days. The results were recorded after four weeks. The experiment was repeated thrice. All cultures were incubated at 25±2ºC with 16/8 hrs photoperiod at 60% relative humidity. Light intensity of 40-50 µmol m^{-2} s^{-1} was provided by using cool white fluorescent tubes.

Extraction of plant material:

The callus material was shade dried, grounded and the powder was kept in small plastic bags with paper labeling. The extraction was done by using soxhlation extraction method with analytical grade methanol as refluxing solvent. After the completion of extraction process, the plant extract was recovered from the mixture by distillation and stored at 4°C until further use. Methanolic extract was used for the phytochemical analysis. The percentage yield of crude methanol extract was calculated by using standard formula (% Yield = Final weight of extract/ Initial weight of extract× 100).

Phytochemical studies:

The prepared extract was tested for different types of chemical constituents present by known qualitative tests.

Test for Phenols:

Ferric chloride test:

About 50 mg of extract was dissolved in distilled water and to this few drops of neutral 5% ferric chloride solution was added. Formation of blue, green and violet color indicates the presence of phenolic compounds.

Test for Tannins:

Lead acetate test:

A small quantity of extract was dissolved in distilled water and to this; 3 ml of 10% lead acetate solution was added. A bulky white precipitate indicates the presence of phenolic compounds.

Test for Flavonoids:

Shinoda test:

A little quantity of extract was dissolved in alcohol and few fragments of magnesium turnings and conc. Hydrochloric acid were added drop wise. Appearance of pink or crimson- red color indicates the presence of flavonol glycosides.

Test for Alkaloids:

About 50mg of solvent free extract was stirred with little quantity of dilute hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloidal reagents as follows.

Mayer's test:

To a few ml of filtrate, two drops of Mayer's reagent was added along with the sides of test tube, appearance of white or creamy precipitate indicates presence of alkaloids.

Test for Carbohydrates:

About 100mg of the extract was dissolved in 5ml of distilled water and filtered. The filtrate was subjected to the following tests.

Molisch's test:

To 2 ml of filtrate, two drops of alcoholic solution of α- napthol was added. The mixture was shaken well and 1 ml of concentrated sulphuric acid was added slowly along the sides of the test tube, the test tube was cooled in ice water and allowed to stand. A violet ring at the junction of two liquids indicates the presence of carbohydrates.

Quantitative phytochemical analysis

Total phenolic content:

The total phenolic content was determined spectrophotometrically by the method described by

Sadasivam and Manickam (1996) after precipitation of proteins. To 2ml of callus extract 1.0 ml of Folin Ceo-calteau reagent was added. After 3 minutes, 13 ml of distilled water was added. Later 2 ml of sodium carbonate (7.5%) solution was added and the volume was adjusted to 20 ml. The above mixture was kept for 1 hour for colour development and absorbance was recorded at 630 nm. The concentration of total phenolic content in callus extracts was calculated from the calibration curve of Gallic acid and it was expressed as Gallic acid equivalents/gram fresh weight. Each experiment has three replicates and the experiment was repeated thrice.

Tannin content:

The tannin content was determined by the method given by Sadasivam and Manickam (1996). To 1ml of callus extract 3.5ml of distilled water and 0.5ml of Folin-Denis reagent was added. Contents were allowed to mix then 1ml of saturated sodium carbonate solution was added. The final volume was made up to 10ml with distilled water. The solution was mixed well at room temperature and the absorbance was measured against prepared reagent blank at 760 nm. Tannin content in plant extracts was calculated from the calibration curve of tannic acid (10-100 µg) and it was expressed as tannic acid equivalents/gram weight. Each experiment has three replicates and the experiment was repeated thrice.

Total flavonoid content:

Total flavonoid content was measured by aluminum chloride colorimetric assay described by Marinova *et al*. (2005). One ml of callus extract was added to 10 ml volumetric flask containing 4 ml of distilled water. To the above mixture, 0.3ml of 5% $NaNO_2$ was added. After 5 minutes, 0.3ml of 10% $AlCl_3$ was added. At 6^{th} min, 2 ml of 1 M NaOH was added and the volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. The total flavonoid content in callus extracts was calculated from the calibration curve of catechin and it was expressed as Gallic acid equivalents/gram fresh weight. Each experiment has three replicates and the experiment was repeated thrice.

Alkaloid content:

Total alkaloid content in the extract was estimated by spectrophotometric method based on using Dragendorff's reagent. The amount of bismuth present was estimated after precipitating the alkaloids with Dragendorff's reagent (Sreevidya and Mehrotra, 2003). Each experiment was repeated three times.

Carbohydrates:

Total carbohydrate content was estimated by Dubois *et al*. (1956). 1ml of extract was taken; 1ml of phenol solution was added. 5ml of 96% sulphuric acid was added to each tube and shaken well and kept in a boiling water bath at 25^{o} C to 30^{o} C for 20 minutes. Absorbance was measured at 490 nm. The amount of total soluble sugars present in the test sample was calculated using a standard graph and the results was expressed as mg/gram extract.

Results:

The percentage of extract after soxhlation was found to be 11.3% for *Sterculia urens.* The phytoactive molecules present in the callus cultures of *Sterculia urens* were qualitatively analyzed and the results were presented in Table 1. The callus extract contains the major secondary metabolites namely phenols, tannins, flavonoids, alkaloids and carbohydrates.

Table: 1. Preliminary screening of callus cultures of *Sterculia urens*

S. No	Name of the phytochemicals	*Sterculia urens*
1	Phenols	+ve
2	Tannins	+ve
3	Flavonoids	+ve
4	Alkaloids	+ve
5	Carbohydrates	+ve

Quantitative analysis of methanolic callus extract of *Sterculia urens* reveals the phenolic content of (132.67±4.81), tannins (44.00±4.16), flavonoids (67.33±3.18), alkaloid (35.00±4.36) and carbohydrate content (66.00±6.43) respectively (Table 2).

Table: 2. Quantitative analysis of phytochemicals in callus cultures of *Sterculia urens*

S. No	Phytochemical	*Sterculia urens* *
1	Phenols (mg/gm)	132.67±4.81
2	Tannins (mg/gm)	44.00±4.16
3	Flavonoids (mg/gm)	67.33±3.18
4	Alkaloids (mg/gm)	35.00±4.36
5	Carbohydrates (mg/gm)	66.00±6.43

***** Each value represents mean ± S.E of three independent experiments and the values were significant at $p<0.05$.

Discussion:

Medicinal plants are reservoirs for traditional and modern medicine, nutraceuticals, pharmaceutical intermediates and chemical entities of synthetic drugs (Ncube *et al*., 2008). Although karaya gum obtained from *Sterculia urens* has enormous medicinal properties, the leaves are also having medicinal importance. Phytochemical screening of methanolic callus extracts derived from leaf showed the presence of phenols, tannins, flavanoids, alkaloids and carbohydrates.

Higher levels of phenols, flavonoids, tannins and alkaloids were observed in callus cultures of *Sterculia urens.* Application of plant growth regulators plays a crucial role in accumulation of plant secondary metabolites in *Sterculia urens*. Similar reports were given by Di-Cosmo *et al*. (1984) and also by Mantell *et*

al. (1984). The enhanced levels of phenols and other metabolites were reported by Nakamura *et al*. (1998), Kaur *et al*. (2009) and Kim *et al*. (2006). Where they reported certain phenols exhibits antiviral, antitumor and anti-inflammatory activities. Similar reports observed during strawberry callus development (Tomas Lopez, 2001). Increased levels of flavonoids during callus cultures were also reported in *Momordica charantia* L by Mala Agarwal and Raka kamal (2007).

Conclusion:

The plants possess a number of pharmacological properties where they are linked to inherent phytochemical constituents. Tissue culture is not only used for mass propagation of plants but also has an advantage of producing valuable secondary metabolites using callus cultures. The concentration of auxins or cytokinins individually or in combination shows an effect on accumulation of phenolics and other compounds in *Sterculia urens*. With this knowledge large scale production of secondary metabolites can be done in future studies.

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