



In-Vitro Propagation of *Dioscorea alata* for Tyrosinase Production

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ABSTRACT

The copper-containing metalloprotein tyrosinase catalyzes the oxidation of tyrosine in particular L-DOPA to L-Dopaquinone that generates brown pigments in the wounded tissues. The industrial demand for tyrosinase enzyme is increasing as they have a wide range of applications in the field of environmental, food, medicine, paper, pulp and textile industry. In the present investigation, tyrosinase was extracted from various plant sources and *Dioscorea alata* having a maximum enzyme activity was chosen. The different parts of plant material were taken for callus development and their enzyme activities were estimated. The enzyme activities of all calli were high comparing to the enzyme extracted from natural tuber tissue of *D. alata* making in-vitro culture as an alternative way for tyrosinase production. It is also evident that 2,4-D has the maximum callus development comparing to other plant growth regulators making it a perfect candidate for proceeding further with media optimization in future.

1. INTRODUCTION

Tyrosinase (E.C: 1:14:18:1) is an enzyme that produces a black pigment by oxidizing tyrosine by means of atmospheric oxygen. Assorted phenols to colored compounds were oxidized by tyrosinase and oxidation products were formed with dihydroxy phenylalanine and pyrogallol. A black pigment called melanin was formed from this dihydroxy phenylalanine, analogous to that formed from tyrosine. The group of catechol oxidase (E.C: 1:10:3:1), laccase (E.C: 1:10:3:2) and tyrosinase were called Poly Phenol Oxidase. This tyrosinase enzyme has a wide range of industrial applications like production of L-DOPA in the field of medicine; detection, quantification and removal of phenolic compounds from water and bioremediation in the field of environmental pollution and production of cross linked polymers in food industry. Because of such an enormous need for the enzyme, development of novel enzyme production methods is needed. Yams are also one of the richest sources of this enzyme and it has been grown in India since very ancient times.

Dioscorea alata commonly called as yam, originated from India was a fundamental food and commercial crop all over the world because of its wealthy carbohydrate and vitamin resource with high calorific value [1]. The genus *Dioscorea* has 600 species with significant economic importance [2] among which 14 are used as edible tubers. India hold a rich genetic diversity of tuber crop especially yam *Dioscorea* [3]. *Dioscorea alata* L. is a significant tuber crop forming a basic food for majority of peoples in subtropical and tropical countries [4]. *Dioscorea alata* is a main crop in West Africa, Caribbean Central and South America [5]. Pulverized tubers were used as a remedy for gonorrhoea, piles and applied externally to sores.

Tubers boast a dual function as they provide nourishment as a food source and also operate as a planting material [6] for vegetative propagation. But numerous virus and fungal diseases impede the tuber production [7]. Standard yam propagation by seeds was sluggish and insufficient for quick reproduction. Nematode and viral infections not only trim down tuber yield radically but also spread to the subsequent generation [8] worsening the tuber quality [9].

Many reasons like deficiency of agronomic constraints and good healthy planting materials along with phytosanitary troubles restrict tuber production. *In vitro* propagation might help to overcome constraints related with availability of high quality of planting material [10] and paved a way to boost the rapid virus free plant material production.

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Tissue culture was an impending way of fabricating handy plant products avoiding all the drawbacks and hindrances in usual agriculture like crop adulteration, disease, environmental factors, handling and storage losses, quality variation, political and labor instabilities in the producing countries. It was an attractive proposal to manufacture important secondary metabolites in bulky bioreactors placed in the using country itself [11].

Callus formation from explants entailed the progressive, more random plane cell division development, prearranged structure loss and less frequent cell specialization [12, 13]. Callus growth can be monitored by fresh weight measurements that are convenient for observing the growth of cultures over time in a non-destructive manner.

In this study, we focused on selecting a plant source for tyrosinase based on its enzyme activity. The chosen plant material was then used for callus development and the enzyme was again extracted from the callus. The enzyme activity from *Dioscorea alata* tubers as well as the callus developed from various parts of *D. alata* was compared to determine the best source for enzyme production.

2. MATERIAL & METHODS

2.1. Plant samples & Chemical requirements

The plant samples like yam, elephant yam and potato were obtained from the agriculture fields close to Sivagangai. Mushroom was obtained from Nila fruits and vegetables, Madurai. Analytical Grade Tyrosine, different pH buffers, distilled water, Sodium Phosphate buffer (pH-7.0) and other general reagents and chemicals used for the present study were purchased from SD–Fine chemicals.

2.2. Tyrosinase Extraction

The tubers of yam, elephant yam and potato were washed with distilled water, skin peeled off and the inner tissue was sliced. Similarly mushroom was sliced into pieces and these sliced plant material were used as enzyme source. About 5 grams of each plant tissue was homogenized with 20 ml of 0.2 M sodium phosphate buffer in separate mortar and pestle. After centrifugation at 5000 rpm for 15 minutes, the supernatant was separated and precipitated with acetone. This precipitate was lyophilized and resolubilized with buffer for further use.

2.3. Tyrosinase Assay

In a clean test tube, 0.5 ml of enzyme extract and 3 ml of 0.1 M Sodium phosphate buffer was pipetted and 1 ml of 1% tyrosine solution was added to it. The enzyme-substrate mixture was immediately transferred into a quartz cuvette and placed in the spectrophotometer. Absorbance values were read at 475 nm for every 30 seconds up to 300 seconds. The absorbance values were recorded and using this absorbance value, the concentration of dopachrome was calculated by Beer-Lambert's law and from that the enzyme activity is expressed in katal (kat).

2.4. Establishment of Aseptic Cultures

Explants collected from field-grown *D. alata* plants are usually contaminated by various microorganisms. The surface contaminants were excluded by washing in the appropriate common sterilizing agents like sodium hypochlorite (1-5%), ethyl alcohol (70-90%) and mercuric chloride (0.1-1%) for 10-30 min followed by several rinses in sterile water. 0.3% Bavistin, 1% benomyl, 0.3-0.5% streptomycin and detergent are also used as surface sterilizing agent.

2.5. Culture Media

A nutrient medium should contain all the necessary major and minor plant nutrient elements, plant growth regulators, vitamins, carbon source and other organic substances as optimal additives. For preparing solid and semi-solid culture media, agar powder is most commonly used. The MS basal medium formulation described by [14] containing 30 g l⁻¹ sugar, 0.1 g l⁻¹ α-cysteine, 10 mg l⁻¹ calcium pantothenic acid, 2 mg l⁻¹ asparagine, 2 mg l⁻¹ arginine and 6 g l⁻¹ agar was used throughout this study for *in vitro* propagation of *Dioscorea alata* [15,16,17]. The medium pH was adjusted to 5.8 prior to autoclaving at 121 °C for 20 minutes.

2.6. Growth Substances

The quantity and type of plant growth regulators incorporated in the culture medium determines the success of tissue culture work. Induction and differentiation of callus and formation of root and shoot were closely synchronized by the relative auxin and cytokinin concentration in the medium [18]. The gibberellins that stimulate callus formation were used as plant growth regulators (PGR) in this study.

2.7. Incubation Condition

The two imperative factors in culture incubation were light and temperature. Cool, white fluorescent lamps providing under 30-50 μmol m⁻² s⁻¹ of light exposure for 12-16 h per day was usually used. The temperature of the culture incubation room was maintained at around 25°C. Due to high phenolic content in yam tissue, mostly it was incubated in darkness to avoid browning.

2.8. Preparation of Explants

Dioscorea alata plants (2 month old) were collected from nearby fields in Sivagangai district and were utilized as explants for plant tissue culture. Explants were thoroughly washed with tap water and then soaked in 0.1% (v/v) of fungicide solution for 10 min followed by sterile distilled water rinsing for four times. Then they were surface sterilized in a viable chlorine bleach containing 4.5% chlorine for 20 minutes and rinsed in a sterile distilled water for four times. Then they were cut into single pieces of specific dimensions.

2.9. Callus Induction from Vegetative Tissues

Stem (5 mm long), leaf (5 mm × 5 mm), corm tuber (5 mm × 5 mm) and petiole (5 mm long) pieces obtained from two month old plantlets from the cultivation fields were evaluated for

callus formation. The treatment media were prepared by dissolving 1 mg l^{-1} of picloram, 2,4-D and NAA separately in MS media. The control tube was prepared by having MS media alone without PGR. The explants were inoculated in glass tube containing 15 ml of treatment medium and incubated in dark at 25°C for 10 days. The most suitable type of explants and PGR was determined based on the highest percentage of callus formation.

2.10. Tyrosinase Extraction from Callus

The callus obtained from all the four types of explants namely nodal stem, leaf, corm tuber and petiole of *D. alata* were sliced aseptically and 10 g of each callus was weighed accurately and minced with a razor blade on a piece of aluminum foil. The Minced callus tissues were transferred to a mortar and 10 ml of 0.2 M phosphate buffer of pH 7 was added and ground to a fine slurry using a pestle. The slurry is then poured onto a small funnel lined with 4 layers of cheesecloth or muslin cloth and strained into a 15 ml screw-top centrifuge tube. The collected filtrate is then balanced and Centrifuged for 15 min. at 3500 rpm. After centrifugation, the supernatant was poured into a fresh 15 ml centrifuge tube, labeled and stored in a fresh bottle in refrigerator for further assay.

3. RESULTS & DISCUSSION

3.1. Selection of Plant Source

The enzyme activity of the crude enzyme extracted from the different plant sources were estimated by continuous spectrophotometric rate determination. The results showed that crude enzyme extracted from *Dioscorea alata* has the maximum enzyme activity of $10588.2 \mu\text{kat ml}^{-1}$ followed by mushroom with next highest enzyme activity (Figure 1).

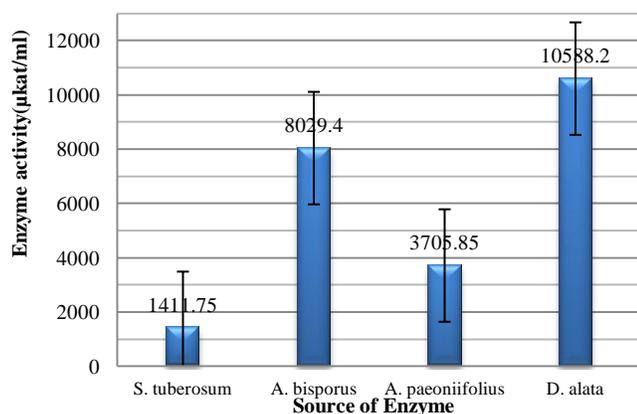


Fig. 1: Comparison of tyrosinase activity from various sources.

3.2. Callus Formation

2,4-D, NAA and picloram at 1 mg l^{-1} induced callus formation from leaf, petiole, corm tuber and nodal stem tissues of *D. alata* after six weeks of culture in the dark. However, these explants did not form callus in the medium without PGR (control). The medium added with 1 mg l^{-1} 2,4-D or picloram induced higher percentage of explants that produced callus compared to 1 mg l^{-1}

NAA. Callus started forming around the edges of the cut area of explant tissue (Fig. 2). Among the four types of explants, the nodal stem produced the highest percentage of purplish nodular embryogenic callus (Fig. 3). Similar results from nodal cuttings were already obtained [19].

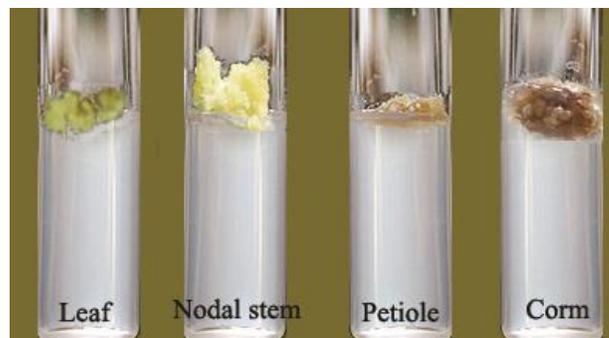


Fig. 2: Callus developed from different explants

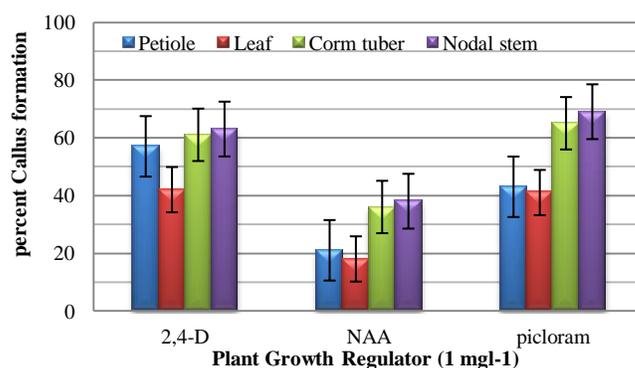


Fig. 3: Percent Callus formation from various explants & PGR

In contrast, the other three explants produced soft fine non-embryogenic callus that were highly vacuolated. More than 50% of the leaf and petiole explants became totally brown and necrotic due to phenolic substances that were exuded from the cut portion [20]. Tissue browning inhibits the production of callus and in severe cases led to the death of tissue [21]. The callus developed from 1 mg l^{-1} 2, 4-D was used for enzyme extraction and assay as they show relatively good percent of callus formation for all explants.

3.3. Tyrosinase Extraction and Assay

The crude enzyme was isolated from the callus tissues and their enzyme activity was estimated by continuous spectrophotometric rate determination. The results showed that crude enzyme extracted from callus developed from corm tuber has the maximum enzyme activity (Fig. 4) even though nodal stem has the highest percentage of callus. Comparatively the enzyme activity from all the callus was much higher than the enzyme isolated from naturally grown tuber tissue of *D. alata*. This clearly depicts that *in vitro* culture can be done for increasing the production of enzyme activity. Also in future the *in vitro* culture

media composition can be optimized using response surface methodology to get more yield of tyrosinase from the callus.

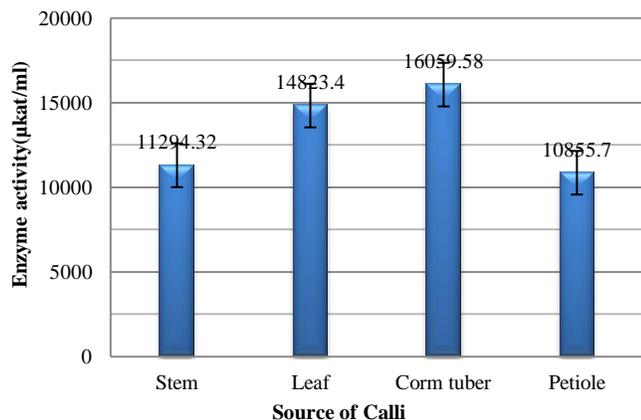


Fig. 4: Comparison of tyrosinase activity from various callus

4. CONCLUSION

Among the four different explants, nodal stem show higher percentage of callus formation whereas the enzymatic activity was higher in callus from corm tuber. Because of the high phenolic content, the calli were brown in color. The enzyme activities of all calli were high comparing to the enzyme extracted from natural tuber tissue of *D. alata* making in-vitro culture as an alternative way for the production of tyrosinase. It was also evident that 1 mg l^{-1} 2,4-D has the maximum calli development comparing to other plant growth regulators making it a perfect candidate for proceeding further with media optimization in future.

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