Peroxidase Activity in Native and Callus Culture of Moringa Oleifera Lam

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***Abstract*—In vitro Moringa oleifera Lam. seedlings were successfully produced through seed culture on Murashige & Skoog (MS) agar medium containing 3% (w/v) sucrose and 0.2% (w/v) Gelrite™ in the absence of growth regulators under 1,500 lux of light density, 16 hour photoperiod light at temperature of 25 ± 2℃. Shoot-derived callus and root-derived callus of M. oleifera were established via culture of shoot and root on the MS medium supplemented with 0.5 mg/l of 2,4-dichlorophenoxy acetic acid (2,4-D) in the dark at 25 ± 2℃. Stem, leaf and root of native M. oleifera and M. oleifera callus were assayed for peroxidase activity using guaiacol as a substrate of the enzyme. In native plant, crude extract from root provided the highest peroxidase specific activity, followed by those from stem and leaf with the specific activity of 19.73 ± 0.18, 16.56 ± 1.43 and 13.38 ± 1.04 unit/mg protein, respectively. Crude extract of root-derived callus and shoot-derived callus of M. oleifera possessed specific activity of 167.25 ± 16.12 and 103.99 ± 10.64 unit/mg protein. These values are significantly higher than their counter parts from native M. oleifera suggesting the potential use of the callus cultures as new and improved sources of peroxidase.**

***Index Terms*—Moringa oleifera Lam., drumstick tree, horseradish tree, callus, peroxidase**

I. Introduction

Moringa oleifera Lam. is commonly known as drumstick tree, horse-radish tree or Ben oil Tree. It belongs to the Moringaceae family and is a native to the sub-Himalaya regions. It is also widely distributed in south-east Asia, tropical Africa and tropical America [1]. All parts of the Moringa tree are edible and have been extensively studied. The leaves are a good source of protein, vitamin C, β-carotene, minerals and antioxidants. The pods are high in fiber [2]. The seeds contain mainly oleic acid (greater than 70%) which can be used in cosmetics and recently considered as a promising source of biodiesel fuel [3]. Flowers are eaten or used to make tea with hypocholesterolemic properties. Bark, gum and root of Moringa oleifera Lam. have multiple medicinal characteristics such as anti-bacterial, anti-fungal and anti-inflammatory [4]. Since *Moringa oleifera* Lam. has been screened for many useful secondary metabolites, we are interested in looking at the availability of useful enzymes. Peroxidase (E. C. 1.11.1.7) is one of the enzymes that have been commercially produced from root of field-grown of horseradish (*Amoracia rusticana*) and soy bean (*Glycine max*) [5]. It is used in clinical diagnosis, immunoassay, nutritional studies, quantitative analysis, organic synthesis for biotransformation of various drugs and waste treatment [6]. Peroxidase is present in bacteria, fungus, animals and plants. Plant peroxidase is distributed in all parts and involved in regulation of plant differentiation and development including responses to hormones and modification of cell-wall properties. It also plays important roles in plant defense mechanism against pathogens and free radicals [7]. Detailed studies have not yet been reported on peroxidase from *M. oleifera*. It is one of our laboratory aims to examine different parts of this plant for potential source of peroxidase. *M. oleifera* which is a perennial tree takes a long time to grow and peroxidase production from the plant may depend on season and growing conditions. Therefore, the production of callus tissue cultures of *M. oleifera* has been considered as another aim for this study. Lastly, the comparative investigation of *M. oleifera* and its callus cultures as alternative sources of peroxidase is reported in this study.

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II. Methodology

A. Preparation of Seedings

Moringa oleifera trees age-1 year obtained from local plant nursery (Chiang Mai province, Thailand) were used for extraction and assay of peroxidase activity in this study. Seeds from dried mature pods of *M. oleifera* from *M. oleifera* tree growing naturally at Chiang Khong district, Chiang Rai province, Thailand were collected as source of the explants. Seeds of M. oleifera were unwrapped from dried pods and thoroughly washed with running tap water. The seeds were surface disinfected with 15% and 30% Clorox solution with 100 µl of Tween 20 for 15 and 30 minutes, respective, followed by four 5 minute rinses in sterile distilled water. Accordingly, the seeds were dipped into 95% ethanol solution and held in a flame for an approximately 30 seconds before the seed coat was carefully removed. The seeds were subsequently germinated on hormone-free sterile Murashige & Skoog (MS) basal agar medium [8] supplemented with 3% (w/v) sucrose and 0.2% (w/v) Gelrite™ (Sigma Chemical Company, USA). The pH of the medium was adjusted to 5.8 before autoclaving at 1.06 kg cm^{-2}, 121℃ for 15 minutes. Seed germination was accomplished under 1,500 lux of light density, 16 hour photoperiod light at room temperature of 25 ± 2℃. Germinated seedlings with healthy roots were used for callus induction.

B. Callus Induction

Shoots and roots of 2 week old *in vitro* seedlings were sliced in uniform size of 0.1 cm^3 and the segments were inoculated separately on MS agar medium, pH 5.8, containing 3% (w/v) of sucrose, 0.2% (w/v) of Gelrite™ supplemented with different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) at 0.25, 0.5, 1.0, 2.0 and 4.0 mg/l. The different concentrations of 2,4-D were tested for callus induction rate reported as percentage callus induction (induction rate (%) = (number of explants producing callus / number of explants cultured) × 100); for 4 weeks for shoot and root explants. All callus cultures were grown in the dark at room temperature of 25 ±2℃. Each experiment had twenty replicates per treatment. The percentages of callus induction were recorded every week. The best medium for callus induction was used for establishment of *M. oleifera* callus cultures and subculturing of callus cultures.

C. Peroxidase Extraction

Different parts of native *M. oleifera* tree (stem, leaf and root) and shoot-derived callus and root-derived callus of *M. oleifera*; were used as the sources for peroxidase extraction. Three different parts of native *M. oleifera* tree; leaf segments (~30g), stems (~70g) and roots (~60g) of the plant were separated and cut in to small pieces then homogenized in a blender with 10 mM phosphate buffer, pH 6.0 (2:1 w/v). The homogenates were filtrated through the cheesecloth twice and centrifuged at 4500xg for 20 min at 4℃. The supernatant obtained was the enzyme crude extracts used for the determination of enzymatic activity and protein content. Eight week old shoot-derived callus or root-derived callus from the *in vitro M. oleifera* seedling grown on the best medium for callus induction were carefully transferred from the medium. The fresh weights of callus for all cultures were recorded before extraction using the same method of that employed for the extraction of peroxidase from different parts of native *M. oleifera* plant.

D. Peroxidase Activity Assay

All enzyme crude extracts were assayed following the method of Agostini *et al.* [9], with slight modification. Peroxidase activity was determined using guaiacol (Sigma, USA) as substrate. The reaction mixture contained guaiacol (15 mM, 1 ml), H_2O_2 (3 mM, 1 ml), phosphate buffer (10 mM, pH 6.0, 950 µl) and enzyme crude extract (50 µl) in a total volume of 3 ml. The reaction was monitored at room temperature by continuously measuring the absorbance at 470 nm for 1 min. All reactions were repeated in triplicate. One unit of peroxidase activity was defined as the amount of enzyme catalyzing the oxidation of 1 µM of guaiacol per 1 min at 25℃, pH 6.0. The activity was calculated using the equation (1) where ΔA/t is the variation of absorbance at 470 nm per min (min^{-1}), Vt is the total reaction volume (ml), Df is the dilution factor, ε is the guaiacol molar extinction coefficient; 26.6 mM^{-1} cm^{-1}, p is the cuvette path length (1cm), Sf is the stoichiometric factor; 0.25 and Sv is the sample volume (µl).

$$Peroxidase\ activity\ (unit/ml) = \frac{\Delta A \times Vt \times Df \times 1000}{t \times \varepsilon \times p \times Sf \times Sv} \quad (1)$$

E. Protein Content Determination

Protein content was determined according to Bradford method [10], using bovine serum albumin as a protein standard.

F. Statistical Analysis

The data was analyzed for variance using Microsoft Excel 2000, followed by a Student's t-test [11].

III. Results and Discussion

A. Preparation of Seedlings and Callus Induction

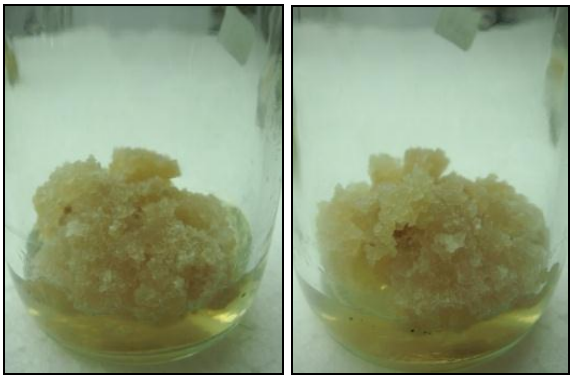
Seed explant germination was observed after 10 days of culture on MS basal agar medium, pH 5.8, without any growth regulators. Complete development of *M. oleifera* plantlets was obtained within 4 weeks after culture initiation as shown in Fig. 1. This suggests that the organic and inorganic components present in the MS medium are sufficient for the production of planlets of *M. oleifera*. The sterile planlets were much more convenient to use for the callus induction as they are free of pathogens, especially those that resides in the root of the native plants. At this stage the shoot segments and root segments of the plantlets were separated and cultured on MS medium supplemented with different concentrations of 2,4-D (0, 0.25, 0.5, 1.0, 2.0, 4.0 mg/l) for callus induction as illustrated in Fig. 2. The synthethic auxin, 2,4-D, was selected for its well known ability to induce callus. However, the suitable concentration for callus induction varies due to the plant species and the nature of tissue of the explants.

Figure 1. *Moringa oleifera* plantlet derived from seed explants on MS agar medium, pH 5.8, without growth regulators.

Figure 2. Pieces of shoot (left) and root (right) of *M. oleifera* plantlets at the time of culture on MS medium supplemented with different concentrations of 2,4-D.

As shoot or root segments of *M. oleifera* plantlet were cultured on MS medium, pH 5.8, free of growth regulators, no callus were present on the explants. Addition of 2,4-D was necessary for the induction of callus of *M. oleifera* from shoot or root explants, as callus were observed for all the explants grown in the culture medium containing selected concentrations of 2,4-D. It was apparent that for the same culture period, the callus derived from shoot explants showed better ability to proliferate on the culture medium than the callus derived from root explants. This is due to the different response of the two plant parts to the same level of 2, 4-D introduced. The *M. oleifera* callus obtained from either type of explants were soft, friable and yellowish-white. The friable characteristic of the callus are suitable for future practice of suspension culture for production of useful secondary metabolites or enzymes. Friable callus derived from shoot and root are shown in Fig. 3(a) and Fig. 3 (b), respectively.

The result of callus induction with 2,4-D in Table I showed that the MS medium supplemented with 0.5 mg/l of 2,4-D was the most effective medium for callus induction of *M. oleifera* with 100% of callus induction from week-3 after culturing of shoot. For the root explants the induction rate reached 68.3±11.7 % in week-3 and 90.3±5.0% in week-4. Not until week-5 did the induction rate become 100%, after culturing of the root segments (data not shown). The explants grown in the MS medium supplemented with 0.25 mg/l of 2,4-D also had high induction rate of 96.7±3.3 % for shoot-derived callus at week-4, while the induction of callus from root segment was only at 61.7±1.7 % at week-4 after inoculation.



(a) (b)

Figure 3. Friable callus derived from shoot (a) and callus derived from root (b) of the *M. oleifera* plantlet cultured on MS medium supplemented with 0.5 mg/l of 2,4-D after 60 days of culturing.

TABLE I. THE INDUCTION OF M. OLEIFERA CALLUS FROM PART OF ROOT OF THE PLANTLETS WITH DIFFERENT CONCENTRATIONS OF 2,4-D (MG/L) SUPPLEMENTED MS AGAR MEDIUM, PH 5.8.

Induction Rate (% ± SE) [a] for Shoot Explants				
2,4-D (mg/l)	Week			
	1	2	3	4
0 (control)	0	0	0	0
0.25	18.3±4.4	48.3±10.1	80.0±5.7	96.7±3.3
0.5	50.0±5.7	88.3±7.2	100	100
1	20.0±7.6	38.3±10.9	53.3±7.2	75.0±7.6
2	13.3±4.4	28.3±4.4	50.0±2.8	65.0±2.8
4	0	11.7±1.6	26.7±4.4	31.7±4.4

Each value represents mean ± SE (Standard error of sample mean) of 20 cultures per treatment in three repeated experiments.

Upon the success of callus induction (week-1) the shoot-derived and root-derived callus were then subcultured at the same time and grown in the MS medium supplemented with 0.5 mg/l of 2,4-D for another 7 weeks. The callus were kept under the same conditions prior to the investigation of peroxidase activity.

B. Peroxidase Activity in Native and Callus Culture of Moringa oleifera Lam

Two types of callus culture of *M. oleifera*, callus derived from shoot and callus derived from root of the plant that were established and subcultured on the MS medium supplemented with 0.5 mg/l of 2,4-D, were examined for perxidase acitivity. Peroxidase activity was also assayed for stems, leaves and roots of native *M. oleifera* tree for comparison with *M. oleifera* callus cultures induced from shoot or root explants. In order to examine the production of peroxidase relative to the other

proteins; specific activity, which is the ratio of peroxidase activity to protein content, was calculated for each crude extract. The data are shown in Table III. In native *M. oleifera* tree, the enzyme crude extract of roots had the highest observed peroxidase specific activity, followed by those of stems and leaves with the enzyme specific activity of 19.73 ± 0.18, 16.56 ± 1.43 and 13.38 ± 1.04 unit/mg, respectively.

TABLE II. THE INDUCTION OF M. OLEIFERA CALLUS FROM ROOT OF THE PLANTLETS WITH DIFFERENT CONCENTRATIONS OF 2,4-D (MG/L) SUPPLEMENTED MS AGAR MEDIUM, PH 5.8.

Induction Rate (% ± SE) [a] for Root Explants				
2,4-D (mg/l)	Week			
	1	2	3	4
0 (control)	0	0	0	0
0.25	6.7 ±1.7	21.7 ±3.3	40.0 ±5.0	61.7 ±1.7
0.5	31.7 ±4.4	50.0 ±10.4	68.3 ±11.7	90.0 ±5.0
1	1.7 ±1.7	16.7 ±4.4	28.3 ±6.0	41.7 ±4.4
2	0	5.0 ±2.9	11.7 ±1.7	16.7 ±4.4
4	0	0	3.3 ±1.7	5.0 ±2.9

Each value represents mean ± SE (Standard error of sample mean) of 20 cultures per treatment in three repeated experiments.

Both types of callus cultures of *M. oleifera* gave significantly higher specific activity of peroxidase than those in the native plant ($p \leq 0.05$). Enzyme crude extract of root-derived callus of *M. oleifera* showed 167.25 ± 16.12 unit/mg of peroxidase specific activity which was higher than that of root of the native plant, approximately 8.5 times higher. This is also notable for the enzyme crude extract of shoot-derived callus which gave the enzyme specific activity of 103.99 ± 10.64 unit/mg which is significantly higher than those of stems and leaves of the native plant for nearly 6.3 and 7.8 times, respectively as shown in Table III.

Moreno *et al.* also investigated peroxidase activity in callus cultures of radish *Raphanus sativus* var. Cherry Bell derived from root, stem and leaf explants. Callus induction was carried with several combinations of benzyladenine (BA) and 2,4-D concentrations in agar MS medium and found that the callus obtained showed significantly greater peroxidase specific activities than those obtained from the native plant [12].

Our data suggest that callus derived from shoots and roots of *M. oleifera* seedling produced peroxidase at a significantly higher level than that produced by the native plant. Peroxidase from the callus will be characterized and evaluated for its potential uses. Moreover suspension culture of *M. oleifera* can now be produced for manufacture of various useful substances which may be found in limited amounts in the native tree.

TABLE III. STATISTICAL ANALYSIS OF P- VALUES FOR NATIVE M. OLEIFERA AND M. OLEIFERA CALLUS CULTURES

Specific activity in plant parts (unit/mg ± SE)	**Stems** (16.56 ± 1.43)	**Roots** (19.73 ± 0.18)	**Leaves** (13.38 ±1.04)	**Callus derived from root** (167.25 ±16.12)	**Callus derived from shoot** (103.99 ± 10.64)
Stems (16.56 ± 1.43)	-	0.159867a	0.148379a	0.011339b	0.014756b
Roots (19.73 ± 0.18)	0.159867a	-	0.027083c	0.011733b	0.015588b
Leaves (13.38 ± 1.04)	0.148379a	0.027083	-	0.010844b	0.013649b
Callus derived from root (167.25 ± 16.12)	0.011339c	0.011733c	0.010844c	-	0.046607c
Callus derived from shoot (103.99 ± 10.64)	0.014756c	0.015588c	0.013649c	0.046607b	-

Statistical analysis was carried out to determine the difference between the specific activity values of the corresponding pair in the vertical and horizontal samples in term of p-value.
The letter "a" indicates no significant different ($p \leq 0.05$ according to t-Test: Two Sample assuming Unequal Variances)
The letter "b" indicates significant decrease ($p \leq 0.05$ according to t-Test: Two Sample assuming Unequal Variances)
The letter "c" indicates significant increase ($p \leq 0.05$ according to t-Test: Two Sample assuming Unequal Variances)

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