Production of Peroxidase from The Cell Suspension Cultures of Horseradish (*Armoracia rusticana*)

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ABSTRACT

Horseradish (HR) plants are considered the main source of peroxidase enzyme. The enzyme is extracted from the roots of field-grown plants which require at least six months to reach maturity. Tissue culture technique was used in the current study as an alternate method to obtain fresh horseradish peroxidase (HRP) throughout the year. The ability of ten cultivars of horseradish (ILH1590, ILH1722, ILH1005, ILH28A, ILH7586, ILH316-3, ILH647, ILH22C, ILH1069 and ILH1038) to produce peroxidase enzyme *in vitro* was investigated. To optimize callus induction, leaf blade, petiole and root segments from the ten cultivars were cultured on MS medium supplemented with 0.5, 0.75, 1 or 5 mg/l 2, 4-D. Virus-free and virus-infected plants of the ten cultivars were used in this study, both grown in either light or dark conditions. HRP activity was determined using spectrophotometric colour changes based on the oxidation of phenol-aminoantipyrene solution in the presence of hydrogen peroxide. In order to standardize the assay, a regression equation was calculated from a standard curve of known HRP activity (Sigma, St. Louis). Activity was expressed in two ways: $\mu mol.min^{-1}.g^{-1}$ FW and as specific activity in $\mu mol.min^{-1}.mg^{-1}$ protein. Protein concentration was determined with a Pierce BCA protein assay kit. The best 2, 4-D concentration used to obtain callus from either leaf blades or petioles was 0.5 mg/l. The results of the current study showed that horseradish cultivars varied in their peroxidase production and that virus-free HR suspension cultures produced more peroxidase than either virus-infected cultures or control. The results also showed that light is essential to maximize the production of peroxidase. In addition the study showed that six weeks old suspension cultures can produce at least as much peroxidase activity as six months old mature field grown roots, thus the *in vitro* system has the capability to speed the manufacture of fresh peroxidase enzyme and facilitate several production cycles through the year.

Key words: horseradish, peroxidase, callus, suspension culture, explants, virusfree plants, cultivars.

INTRODUCTION

Horseradish (*Armoracia rusticana* P. G. Gaertn., B. Mey. *et* Scherb.) is a perennial crop that belongs to family Brassicaceae. Horseradish roots are a rich source of the peroxidase enzyme Kushad *et al.*, (1999). Peroxidase (present in peroxisomes) prevents the toxic accumulative effects of hydrogen peroxide (H_2O_2), a strong oxidizing agent produced as an end product of oxidative metabolism (Oneonta University, 2004). HRP is a well-known enzyme used as an indicator for various chemical reactions that produce peroxide. The enzyme is used within enzyme-based immunoassay systems by conjugating it to antibodies. Peroxidase has been also used in bio-bleaching Harazono *et al.*, (1996), in degrading of wood pulp into fuel (Macek *et al.*, 1993), and in removal of carcinogenic pollutants from industrial wastewater Peive *et al.*, (1972); (Klibanov and Morris, 1981); (Dec and Bollag, 1994); Roper *et al.*, (1996;). (Krel 1991) reported that the world production of horseradish peroxidase was estimated by 30 billion kilo-units, and he expected demand to double in 2010. The three major HRP producers in the United States are Sigma[1] Chemical Co., St. Louis; ICN Pharmaceuticals[2] Inc., Costa Mesa, California[3] and Boehringer Mannheim Corp., Indianapolis, Indiana Kushad *et al.*, (1999). Peroxidase is present in various plants, however, the highest known concentration is found in the roots of horseradish (Scripps laboratories, 2003). Although, peroxidase activity has been reported in peanut, tobacco, and soybean (Buttery and Buzzell, 1968); Cairns *et al.*, (1980; Lobarzewski and Van Huystee, 1982), the highest peroxidase activity in soybean seed is about 1% of that in horseradish Kushad *et al.*, (1999).

Horseradish roots are the most economically important part of the plant and are the source for the enzyme peroxidase. HRP is usually harvested from fresh field-grown HR roots. *In vitro* culture could provide an alternate method to obtain fresh HRP throughout the year. In this study, the yields of HRP from suspension cultures were compared to those of fresh roots.

MATERIALS AND METHODS

This research was carried out at the University of Illinois at Urbana – Champaign in 2008. The ability of ten cultivars of horseradish (ILH1590, ILH1722, ILH1005, ILH28A, ILH7586, ILH316-3, ILH647, ILH22C, ILH1069 and ILH1038) to produce peroxidase enzyme *in vitro* was investigated.

Preparing plant material.

For these experiments *in vitro* plants were produced in two ways: from field grown (mosaic

virus-infected) plants and from *in vitro* cultures that had previously been freed from virus (virus-free) by meristem cultures. For the virus-infected group, root cuttings of the ten cultivars were harvested from the University of Illinois horseradish germplasm collection and planted in pots in a greenhouse where they grew to form new plants. Six weeks later after planting, vigorous new leaves were harvested from these plants for *in vitro* studies. Leaf segments were disinfested with 10% bleach (0.6% NaOCl) and 0.1% Tween for 10 minutes on a shaker (100 rpm), rinsed with sterile distilled water three times, five minutes per rinse, cut into 1.5 cm^2 pieces, and transferred onto Murashige and Skoog (1962, MS) medium supplemented with naphthalene acetic acid (NAA, 15 µmol l^{-1}) in Petri dishes (100 mm diameter, ca. 30 ml/plate) using the procedures of Norton *et al.* (2001) and Shehata *et al.* (2009).

Virus-free cultures were established from leaves of virus-free plants harvested from *in vitro* plants and cultured on the same medium described earlier. Six weeks after transfer, shoots were harvested from both virus-infected and virus-free plants and placed on MS medium and allowed to elongate for another three weeks.

Callus induction studies.

To identify an optimum medium and explant for callus induction, leaf blade, petiole and root segments were harvested from all *in vitro* cultivars, both virus-infected and virus-free, and transferred to MS medium supplemented with 2, 4-D for six weeks. Parkinson *et al.* (1990) reported good horseradish callus growth at 5 mg/l 2, 4-D. However, it has been found in our laboratory that lower 2, 4-D concentrations were useful. To investigate this, a range of 2, 4-D values were examined; concentrations of 0.5, 0.75, 1 and 5 mg/l 2, 4-D were used. Each treatment consisted of five replicates (Petri dishes) and each replicate contained five explants in a completely randomized design (CRD). Diameter of callus produced from each explant of the ten cultivars was recorded in cm after six weeks *in vitro*.

Peroxidase production studies.

To study the production of peroxidase enzyme, one gram of callus was taken from each cultivar, macerated and placed in 30 ml liquid MS medium supplemented with 0.5 mg/l 2, 4-D and placed on a shaker (100 rpm) for six weeks. The jars were either grown in light or were wrapped with aluminum foil to insure darkness. All jars were incubated in a culture room maintained under 16-h days (cool white fluorescent light) between 20 and 22°C. The photosynthetically active radiation (PAR) at the level of the medium surface was 131 µmol $m^{-2}s^{-1}$. The peroxidase content of each treatment was assessed at the end of a six week incubation period.

To compare the peroxidase activity of cell suspensions and whole plants (control), a sample from the suspension cultures of all ten cultivars was taken from each cultivar (virus-infected and virus-free) grown in light or dark. The sample was prepared by using a mortar and pestle to grind all cells and supernatant to slurry. A 100 µl sample was taken for analysis. For control, field-grown plants were used. A root segment (about 3 cm long) was taken from the middle portion of the three largest roots of each plant. The root segments were cleaned with distilled water, macerated using a hand chopper, and then combined. One gram fresh weight of the mixture was placed in a mortar and ground with a pestle in the presence of 6 ml of extraction buffer consisting of 0.5 M calcium chloride solution. The slurry was centrifuged at 1000 x gravity for eight minutes. The supernatant containing peroxidase was collected in a clean labeled test tube and stored on ice. The peroxidase extraction and activity determinations used in our studies were done following the procedures outlined by the United States Environmental Protection Agency (EPA, 1994).

HRP activity was determined using spectrophotometric colour changes based on the oxidation of phenol-aminoantipyrene solution in the presence of hydrogen peroxide in buffer solution [0.01 M 2-(N-Morpholino) ethanesulfonic acid or MES solution] at pH 6.0 Kushad *et al.*, (1999). The reaction mixture consisted of 1.4 ml of phenol-aminoantipyrene solution (16.2 mg phenol and 0.5 mg 4-aminoantipyrene per ml), 1.5 ml of 0.3% hydrogen peroxide in 0.01 M MES buffer solution (pH 6.0) and 100 µl enzyme extract.

The mixture was placed in a 3-ml cuvette and the optical density was recordered by measuring the change in the initial and final absorbance at 510 nm for two minutes using a Shimadzu UV160U spectrophotometer (Shimadzu Corp., Kyoto, Japan). In order to standardize the assay, a regression equation was calculated from a HRP standard curve Figure (1).

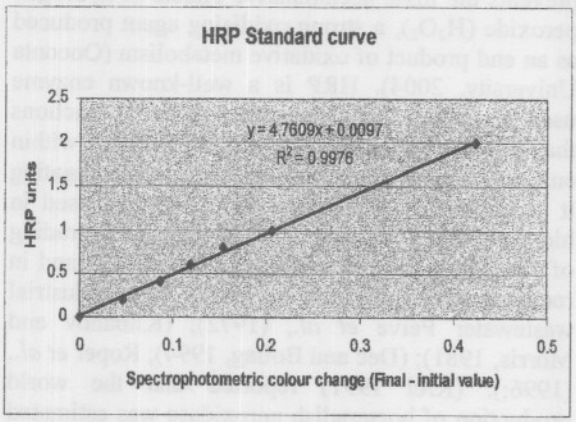

Fig. 1: Horseradish peroxidase standard curve calculated by using HRP of known activity (Sigma, St. Louis) dissolved in extraction buffer and assayed in the same manner as the unknown samples using spectrophotometer.

The standard curve was calculated by using HRP of known activity (Sigma, St. Louis) dissolved in extraction buffer and assayed in the same manner as the unknown samples.

Activity was expressed in two ways: $\mu mol.min^{-1}.g^{-1}$ FW and as specific activity in $\mu mol.min^{-1}.mg^{-1}$ protein. Protein concentration was determined with a Pierce BCA protein assay kit using bicinchoninic acid (BCA). Each treatment consisted of 12 replicates in a completely randomized design (CRD). Peroxidase activity and protein concentration were recorded after six weeks *in vitro*. Data were statistically analyzed by the analysis of variance (ANOVA) using GLM procedure in the SAS software package (SAS Institute, 1999) and the differences among treatment means were compared using LSD.

RESULTS AND DISCUSSION

Results obtained from the current study showed that all explants used *in vitro* were clean and free from any contamination and that surface disinfestation was affective and no visual contamination was detected.

Callus induction studies.

Leaf blade segments and petioles formed callus equally well on media with less than 5 mg/l 2, 4-D Figure (2). None of the explants survived the highest level of 2, 4-D (5 mg/l). The best 2, 4-D concentration used to obtain callus from either leaf blades or petioles was 0.5 mg/l Table (1). However, Mevy *et al.* (1997) reported that 0.2 mg/l 2, 4-D produced callus from horseradish leaves in German cultivars of horseradish. Shigeta and Sato (1994) produced callus from horseradish leaves using concentrations of 2, 4-D that ranged from 0.1- 2.0 mg/l. Parkinson *et al.* (1990) reported that 5 mg/l 2, 4-D was optimal for horseradish callus induction. In the current study, root segments failed to form callus, regardless of 2, 4-D concentrations.

The fact that the investigated cultivars grew better callus at relatively low levels of 2, 4-D (0.5 mg/l) suggests that there are large differences among horseradish cultivars for *in vitro* growth requirements.

Fig. 2: Callus growth from horseradish leaf petiole after six weeks *in vitro* on MS medium supplemented with 0.5 mg/l 2, 4-D

Peroxidase production studies.

Cultivars varied in their ability to produce peroxidase *in vitro* Table (2) which agrees with the findings of Kushad *et al.* (1999). Based on their HRP activity cultivars were divided into three categories: High activity which included ILH 28, ILH 647, ILH 1005, ILH 1722 and ILH1069, Moderate activity included ILH 1590, ILH 1038 and ILH 22C and Low activity included ILH 7586 and ILH 316-3. The cultivars ILH 28A and ILH 647 contained the highest activity (148.53 and 145.36 $\mu mol.min^{-1}.g^{-1}$ FW, respectively) and cultivar ILH 316-3 contained the lowest activity (47.98 $\mu mol.min^{-1}.g^{-1}$ FW).

The cultivar ILH 1590 contained the highest activity on a unit protein basis (35.29 $\mu mol.min^{-1}.mg^{-1}$ protein), followed by ILH 647 and ILH 1722 Table (2). The lowest specific activity was found in cultivar ILH 316-3 (7.46 $\mu mol.min^{-1}.mg^{-1}$ protein). Protein concentrations were also variable among the cultivars Table (2). Cultivar ILH 1069 had the highest protein concentration (9.9 mg. g^{-1} FW) followed by ILH 22C and ILH 316-3 Table (2). The lowest protein concentration was found in cultivar ILH 647 (5.78 mg. g^{-1} FW).

The activity and specific activity of virus-free plants were significantly higher than either virus-infected or the control samples Table (3). The highest activity was found in virus-free cells ($129.24 \mu mol.min^{-1}.g^{-1}$ FW) compared to virus-infected and control samples (98.34 and $97.75 \mu mol.min^{-1}.g^{-1}$ FW respectively). The highest specific activity was also found in virus-free cells ($33.66 \mu mol.min^{-1}.mg^{-1}$ protein) compared with virus-infected and control samples (21.46 and 6.20 $\mu mol.min^{-1}.mg^{-1}$ protein respectively). The significantly higher specific activity found in *in vitro* suspension cultures (both virus-free and virus-infected) was due to their relatively low protein concentration compared to control plants Table (3). Macek *et al.* (1993) reported that HRP activity was four fold higher in the supernatant of suspension culture as in cells or callus and 30-fold as high as in root tissue. Khadeeva *et al.* (1993) reported that horseradish tissue culture can be used as a source for peroxidase production. They found that the enzyme content obtained from callus was equal or higher than that in field grown roots. Kushad *et al.* (1999) reported that horseradish root age was not a limiting factor in horseradish and that roots harvested at different time of the year were significantly the same in their HRP activity.

Suspension cells grown in light had significantly higher activity and specific activity values than either dark-grown cells or control plants Table (4). The highest specific activity of light-grown suspension cultures ($37.81 \mu mol.min^{-1}.mg^{-1}$ protein) resulted from the relatively low protein concentration (3.98 mg. g^{-1} FW) compared to control plants (18.90 mg. g^{-1} FW) Table (4). The results also showed that cells grown in the dark had

Table 1: Diameter of callus in cm produced at different concentrations of 2, 4-D from leaf blades and petioles of ten horseradish cultivars after six weeks *in vitro*

Explant	2,4-D concentration (mg/l)				Explantsmean
	0.5	0.75	1 .00	5.00	
Leaf blade	1.86 a*	1.63 b	0.99 c	–[z]	1.49 b
Leaf petiole	1.82 a	1.59 b	0.94 c	–	1.45 b
Root segment	–	–	–	–	–
2,4-D mean	1.84 a	1.61 b	0.97 c	–	1.47

* Values with different letters are significantly different by $FLSD_{.05}$

[z] = No callus growth (excluded from mean separation)

Table 2: Peroxidase activity, specific activity and protein concentrations of suspension cultures of ten horseradish cultivars on Murashige and Skoog tissue culture medium

Cultivar	Activity ($\mu mol.min^{-1}.g^{-1}$ FW)	Specific activity[z] ($\mu mol.min^{-1}.mg^{-1}$ protein)	Protein ($mg.\ g^{-1}$ FW)
ILH 28 A	148.53 a*	29.34 ab	7.97 b
ILH 647	145.36 a	31.92 ab	5.78 d
ILH 1005	137.56 ab	25.19 bc	8.45 b
ILH 1722	134.79 ab	31.6 ab	8.02 b
ILH 1069	119.82 abc	20.27 dc	9.9 a
ILH 1590	112.54 bc	35.29 a	6.26 dc
ILH 1038	96.58 dc	20.59 dc	7.46 bc
ILH 22 C	94.4 dc	15.75 de	8.74 ab
ILH 7586	68.28 ed	15.48 de	7.79 b
ILH 316-3	47.98 e	7.46 e	8.63 ab

* within columns, values with different letters are significantly different by $FLSD_{.05}$

[z] Specific activity = Activity/ Protein

Table 3: The effect of virus status (Mosaic virus) on the activity, specific activity and protein concentrations of 6 week-old HR suspension cultures of ten horseradish cultivars

	Activity ($\mu mol.min^{-1}.g^{-1}$ FW)	Specific activity[z] ($\mu mol.min^{-1}.mg^{-1}$ protein)	Protein ($mg.\ g^{-1}$ FW)
Virus-free	129.24 a*	33.66 a	4.82 b
Virus-infected	98.34 b	21.46 b	5.48 b
Control[x]	97.75 b	6.20 c	18.90 a

* within columns values with different letters are significantly different by $FLSD_{.05}$

[z] Specific activity = Activity/ Protein

[x] control = root segments harvested from 6 months old mature HR roots grown under field conditions

Table 4: The effect of light and darkness on the activity, specific activity and protein concentrations measured from suspension cultures of ten horseradish cultivars after six weeks *in vitro*

	Activity ($\mu mol.min^{-1}.g^{-1}$ FW)	Specific activity[z] ($\mu mol.min^{-1}.mg^{-1}$ protein)	Protein ($mg.\ g^{-1}$ FW)
Light	130.17 a*	37.81 a	3.98 c
Darkness	97.41 b	17.31 b	6.32 b
Control[x]	97.75 b	6.20 c	18.90 a

* within columns values with different letters are significantly different by $FLSD_{.05}$

[z] Specific activity = Activity/ Protein

[x] control = root segments harvested from 6 months old mature HR roots grown under field conditions

more protein concentration (6.32 mg. g^{-1} FW) than cells grown in the light (3.98 mg. g^{-1} FW).

The results suggest that virus-free suspension cultures grown in light would yield more peroxidase activity and produce significantly more enzyme than either virus-infected or control plants. Yamada *et al.* (1987) reported that peroxidase extracted from cultured cells was equivalent in its performance to the commercially available peroxidase. They also reported that peroxidase activity and growth rate are two important factors among cultivars selected for commercial production of peroxidase by plant cell techniques. Parkinson *et al.* (1990) reported that cell suspension cultures produced twice the total activity of root hair cultures and that peroxidase activity is critically related to maximizing growth rate. And since light is a limiting factor required for plant growth, this explains our findings that cells grown in the light had significantly higher enzyme activity than those growing in the dark. However, the situation with HR tuberous roots might be different, where it was reported that Sudhir and Mukundan (2002) produced peroxidase enzyme from tuberous horseradish roots *in vitro* from cultures grown in dark.

In Summary, the results of the current study showed that horseradish cultivars varied in their peroxidase production and that virus-free HR suspension cultures produced more peroxidase than either virus-infected cultures or control (field-grown plants). The results also showed that light is essential to maximize the production of peroxidase. In addition the study showed that six weeks old suspension cultures can produce at least as much peroxidase activity as six months old mature field grown roots, thus the *in vitro* system has the capability to speed the manufacture of fresh peroxidase enzyme and facilitate several production cycles through the year.

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الملخص العربى

إنتاج البيروكسيديز من مزارع الخلايا المعلقة لنبات فجل الحصان

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إجرى هذا البحث فى جامعة إيلنوى بالولايات المتحدة الأمريكية سنة 2008 وذلك بهدف دراسة إنتاج إنزيم البيروكسيديز من مزارع الخلايا المعلقة لنبات فجل الحصان علي مدار العام. والمعروف ان نبات فجل الحصان يعتبر المصدر الرئيسى لإنزيم البيروكسيديز الذي يتم إستخلاصه من جذوره والتي تحتاج لأكثر من ستة اشهر من الزراعة في الحقل لكي تصل الي مرحلة النضج . وفي هذا البحث تم دراسة قدرة عشرة أنواع من نبات فجل الحصان علي إنتاج البيروكسيديز من مزارع الخلايا المعلقة. ولقد مر البحث بثلاثة مراحل للوصول إلى هدفه:

1. إنتاج الكالس:

حيث زرعت أجزاء من نصل الورقة وأجزاء من عنق الورقة وأجزاء من الجذور علي بيئة غذائية صلبة (MS) تحتوي علي تركيزات 0.5 أو0.75 أو 1 أو 5 مجم / لتر من ال 2,4-D وذلك بهدف الوصول الي أفضل الأجزاء النباتية والتركيز الأمثل من2,4-D لإنتاج الكالس.

2. دراسة تأثير الإصابة بفيرس الموزيك علي إنتاج البيروكسيديز من مزارع الخلايا المعلقة.

حيث تم مقارنة إنتاج الخلايا المأخوذة من نباتات خالية من الفيرس بأخرى مأخوذة من نباتات مصابة ثم مقارنة كليهما بإنتاج الجذور في الحقل (كونترول).

3. دراسة تأثير الضوء والظلام علي إنتاج البيروكسيديز من مزارع الخلايا المعلقة.

حيث تم مقارنة إنتاج الخلايا النامية فى الضوء بأخرى نامية فى الظلام ثم مقارنة كليهما بإنتاج الجذور في الحقل (كونترول).

و قد أظهرت نتائج البحث أن أفضل إنتاج للكالس كان عند تركيز 0.5 مجم /لتر 2,4-D وذلك بإستخدام أجزاء من الأوراق أو الأعناق.

كما أظهرت النتائج أن إنتاج البيروكسيديز من مزارع الخلايا المعلقة إختلف إختلافا معنويا بإختلاف أنواع فجل الحصان و أن إنتاج الإنزيم من الخلايا الخالية من الفيرس كان أكثر من الخلايا المصابة بالفيروس و أن تعريض مزارع الخلايا المعلقة للضوء أدي إلى زيادة إنتاج الإنزيم منها، كما أظهرت النتائج أيضا أن خلايا عمرها ستة أسابيع في مزارع الخلايا المعلقة أمكنها إنتاج نفس كمية البيروكسيديز علي الأقل التي تنتج بعد ستة اشهر من الجذور المنزرعة بالحقل. ولذلك فإن إستخدام تكنيك مزارع الخلايا المعلقة يمكن أن يؤدي إلى إنتاج عدة دورات إنتاجية من إنزيم البيروكسيديز خلال العام.