Natural Products Chemistry & Research

ISSN: 2329-6836

Sharma et al., Nat Prod Chem Res 2018, 6:6
DOI: 10.4172/2329-6836.1000347

Research Article Open Access

Saline Resistance Enzyme Estimation in *Mentha piperata* L.

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Received: November 12, 2018; **Accepted:** November 27, 2018; **Published:** November 30, 2018

Abstract

During the present set of work, various activities like Peroxidase, IAA-oxidase, Polyphenol and Ascorbic acid oxidase were studied in *Mentha piperata L*. Peroxidase activity and IAA oxidase activity were found to be higher in *in vitro* plants than *in vivo* plants, which indicate that *in vitro* plants have superior enzyme activity. However, Polyphenol activity was negligible in *in vitro* plants whereas *in vivo* plant showed higher activity. There was very little difference in ascorbic acid oxidase activity of both *in vitro* and *in vivo* plants. The peroxidise enzyme and IAA oxidase can give the strength to the plant to survive in saline environment also, so if these increase in *in-vitro* condition so if they will hardened and grows in saline condition also, so by this we can shows the high vegetation in saline climate also.

Keywords: Peroxidase; IAA-oxidase; Polyphenol ascorbic acid oxidase; *Mentha piperata*; *In vitro* and *In vivo* plants

Introduction

The term biochemistry was introduced by Carl Neuberg. Biochemistry broadly deals with the chemistry of life and living processes. The scope of biochemistry is vast as life itself. Every aspect of life, birth, growth, reproduction, ageing and death involves biochemistry. The goal of biochemistry is to understand the chemical basis of biological phenomenon. It is the most rapidly developing and innovative subject in medicine. Moreover, in the area of medicinal biology, natural products or their designed analogues were used for the study of novel therapeutic agents. Although they have been directly used as food, fibre and medicine, yet these plants are the most important source of life saving drugs for the majority of the world's population [1]. The biochemistry serves as a torch of light to solve the intricate complexities of biology besides unravelling the chemical mysteries of life.

Biochemical research has amply demonstrated that all living things are closely related at the molecular level. Advances in biochemistry have led to tremendous impact on human welfare and their living styles. These include the application of biochemistry for the diagnosis of diseases, the products (Insulin, interferon, grown hormone etc.,) obtained from genetic engineering and the possible use of gene therapy in the near future. In this aspect, enzymes also play a very important role, they are the proteins that facilitate biochemical reactions.

Enzymes are proteins that are catalysts for chemical reactions. From Chemistry point of view, it is known that catalysts are non-consumable substances that reduce the activation energy necessary for a chemical reaction to occur. Enzymes are highly specific to the reactions they catalyze. They are of vital importance for life because most chemical reactions of the cells and tissues are catalyzed by enzymes. In biological processes, reactions would not occur in the required speed without enzymatic action in which they participate e.g., include the enzymes lactase, pepsin, rennin etc.,

The interaction between phenolic compounds and protein with reference to the inhibition of enzyme activity has been reviewed by Loomis and Bottaile [2]. According to them phenolic compounds can bind to proteins by hydrogen bonding or may be oxidized to quinines which co-polymerizes with protein through covalent bonding. Quinones may also condense to form tannins or brown pigments which inactivate enzymes and may cause precipitation of soluble proteins. Numerous endogenous phenolic compounds are recognized as protein precipitants [3] and enzyme inhibitors [4]. Various additives to an extraction medium have been employed to remove these inhibitors. These include polyethylene glycol as reducing agents both soluble and insoluble like Poly vinyl pyrrolidone [2] and borate [5]. In all these cases, a number of problems crop up, for example it is not possible to remove PEG from proteins by gel filtration or dialysis. PVP (both soluble and insoluble) and borate have proved moderately successful in removing these inhibitors and they adversely affect some enzymes [5]. Furthermore, the dialysis of these substances is again a cumbersome process. Therefore, their use in everyday work is most inconvenient. In the present work chilled acetone for precipitation of proteins was found to be most suitable and therefore enzyme extracts were purified by chilled acetone precipitation.

Peroxidase and IAA oxidase enzyme and plant environment

Peroxidase enzyme and IAA oxidase gives the resistance and tolerance power to the plant to survive in saline environment. If these enzymes is elevating in the plant body the plant can survive at high salinity, thus we say if we increase these enzyme then if environment or water which is used by palnt to grow have high survival rate [6].

Materials and Methods

Sample preparation

Complete plantlets (*In vitro* and *In vivo*) as source of sample preparation were used for various enzymatic activities in *M. piperata L.* 1.0 gm plant material was weighed and homogenized with 3 ml of chilled phosphate buffer (0.02 M, pH 6.4) in pre-cooled mortar and pestle. It was centrifuged at 10000 rpm for 15 minutes in cooling

centrifuge (Remi-K-24) at 4°C. The supernatant was mixed with the double amount of chilled acetone and incubated at 50°C for half an hour for precipitation of the soluble proteins. It was re-centrifuged at 3600 rpm for 10 minutes at 40°C. The supernatant was discarded while residue (precipitated protein) was resuspended in 10 ml phosphate buffer (0.02 M, pH 6.4) and used as enzyme source.

Peroxidase activity

1.0 ml of enzyme aliquot was mixed with 1.0 ml of phosphate buffer (0.1 M, pH 6.4) and 1.0 ml of 20 mM guaicol. After that 0.5 ml of H_2O_2 was added and optical density was recorded at 420 nm after every 2 mins. Calculation was done and expressed as optical density increased/minute/gm plant material. Blank was prepared in the same manner.

IAA oxidase activity

Three test tubes were taken for this activity (1) test (2) control (3) blank. 1.0 ml of enzyme aliquot, 1.0 ml of indole-3 acetic acid (IAA) stock solution as standard solution, 1.0 ml of manganese chloride ($MnCl_2$) (0.5 mM), 2,4-dichloro phenol (DCP) (0.1 mM) and 1 ml of phosphate buffer (0.05 M) were added to the first test tube. 1.0 ml DW, 1.0 ml IAA stock solution, 1.0 ml $MnCl_2$, 1.0 ml DCP and 1 ml phosphate buffer were added to the second (control) test tube. 2.0 ml DW, 1.0 ml $MnCl_2$, 1.0 ml DCP and 1.0 ml phosphate buffer were added to the third (blank) test tube. All the three test tubes were incubated in the dark for one hour at room temperature (25°C). 2 ml solution from each test tube was taken and 2.0 ml Salkowoski's reagent (SWR) was added to each. Again all test tubes were incubated for 30 minutes in the dark at room temperature. Optical density was recorded at 530 nm. Blank was related with zero setting, control gave the reading of pure IAA, while test gave the reading of oxidized IAA. Calculations were done by using the readings and result were expressed as mg IAA oxidized/gm plant material.

Poly phenol oxidase (PPO) activity

1 ml of enzyme aliquot was mixed with 2 ml of phosphate buffer (0.02 M, pH 7) and 2 ml of 50 mM pyrogallol solution. It was incubated at room temperature (25°C) for 2 minutes and optical density was recorded at 420 nm. This activity was calculated and expressed as optical density/2 minutes/gm plant material.

Ascorbic acid oxidase

Plant material was homogenized in 10 ml of 0.5 M phosphate buffer (pH 6.0) and centrifuged at 5000 rpm at 4°C for 10 minutes. The dialyzed supernatant was taken for enzyme assay. 0.2 ml of the enzyme extract, 1.0 ml of the phosphate buffer (pH 6.0), 1.6 ml of distilled water and 0.2 ml of 0.1 M ascorbic acid was added. The absorbance was recorded at 265 nm after each 15 sec. intervals up to 3 minutes. Cuvette containing buffer was used to adjust the absorbance to zero. The enzyme extract was expressed in terms of change in absorbance/sec./mg protein.

Results and Discussion

During the present set of experiments, isozyme activity was estimated in complete plantlet (*In vitro* and *In vivo*) of *Mentha piperata L.* Peroxidase activity was found to be higher in *In vitro* plants than *In vivo* plants (Figure 1). Likewise, IAA oxidase activity is higher *In vitro* plants as compared to *in vivo* plants. Thus we can conclude that *in vitro* plants show good enzyme activity (Figure 2). Poly phenol activity was negligible in *in vitro* plants, whereas *in vivo* plant showed higher activity (Figure 3). There is very little difference in ascorbic oxidase activity of both *in vitro* and *in vivo* plants (Figure 4).

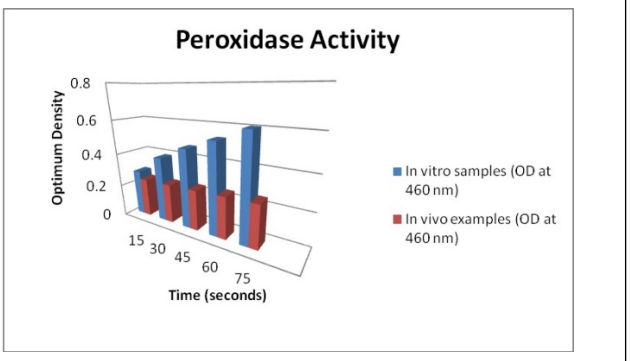

Figure 1: Peroxidase Activity.

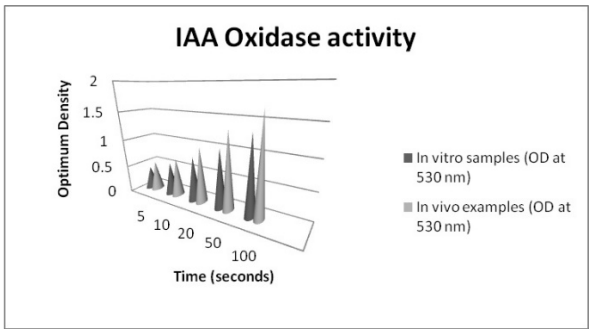

Figure 2: IAA oxidase Activity.

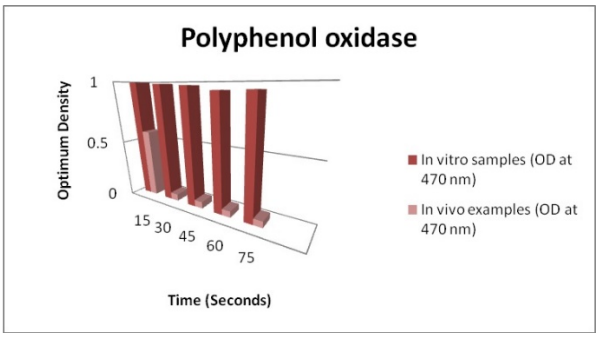

Figure 3: Polyphenol oxidase.

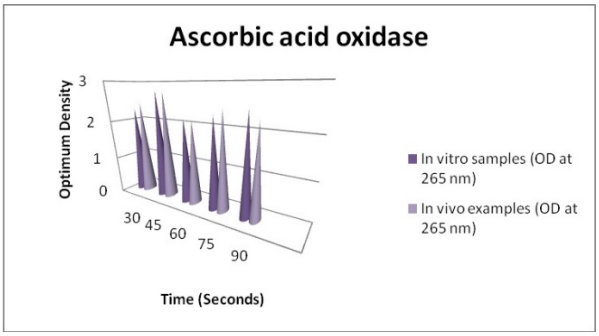

Figure 4: Ascorbic acid oxidase.

Peroxidases play a significant role in the growth and differentiation of higher plants. Peroxidises are present in almost every plant tissue and their levels and isozymes vary with developmental events [7]. Even though they have not been definitely involved in specific metabolic pathways, their tissue levels have been negatively correlated to plant growth and IAA levels [8], while positively correlated to lignin synthesis. Malik and Singh suggested that peroxidases are widely distributed in plant tissues and are of immense physiological interest because of their association with numerous catalytic functions [9]. Although, variation in peroxidase activity was associated with disease resistance, loss of pollen and seed viability, its activity was particularly associated with auxins besides growth and differentiation. However, alteration in peroxidase activity has been reported in certain induced mutants in crop plants [10,11]. Furthermore, peroxidases have often served as a parameter for metabolic activity during growth alterations and comprises of a family of several isozymes. Plants have a large number of peroxidase enzymes that may differ by more than 50% in amino acid sequences. Plant peroxidases are among the most studied but least understood of all plant proteins [12].

However, several factors like mineral nutrition, cold temperature hardening, disease hardening, disease infection and injury affect the intensity of isozymes. Several researchers have carried out studies for observing changes in the activity and isozymes of peroxidase in *in vitro* conditions [13,14]. In rice, Futsuhara have observed the appearance of new peroxidase isozyme bands and increased ability for shoot bud differentiation, it shows their close correlation to each other [15].

Isozyme modifications have been observed by Chawla during morphogenesis of callus from barley and wheat and it was observed that with the development of shoots and roots some conspicuous isozymes disappeared [16]. Peroxidase isozymes pattern have been observed in the skin of maturing tomato fruit [17,18]. It is concluded that the late-appearing isozymes are not associated with fruit ripening or softening and are probably not ethylene-induced. They may act to control fruit growth by cross-linking wall polymers within the fruit skin, thus mechanically stiffening the walls and terminating growth. In vitaceae, a general pattern for peroxidase isozyme and their functions have been studied. In vitaceae pattern can be used as biochemical marker for rooting and multiple shoot initiation in cocoyam.

Peroxidase and catalase changes during *in vitro* adventitious shoot organogenesis from hypocotyls of *Albizia odoratissima* L.f. (Benth) were studied by Rajeswari and Paliwal [19]. The changes in the specific peroxidase and catalase activity, total protein content and acidic isoperoxidase pattern were compared between the cultures showing shoot organogenesis and cultures producing non-morphogenic calli. It was found that *in vitro* shoot bud differentiation is accompanied by an increase of the specific activities of peroxidase and catalase in culture kept under light. Conversely, culture producing non-morphogenic calli underwent a reverse change in specific peroxidase activity. This change in antioxidant enzyme activities corresponds to the histological observation of shoot bud differentiation in cultures kept under light.

Polyphenol oxidase (PPO) occurs widely in nature in higher plants and in microorganisms. Polyphenol oxidase is variously known as DOPA oxidase, tyrosinase, catechol oxidase and potato oxidase. Poly phenol oxidase performs the function of catalyst in the oxidation of o-diphenols and p-phenols. It is reported to catalyze the biosynthesis of many plant products [19] and synthesis of IAA. There was a positive relation between PPO activity and the occurrence of protein-bound phenols with a concomitant decrease in measured lipase activity, indicating a possibility to a direct inhibition of enzymes as a result of protein-bound phenols [20].

Changes in morphology and biochemical indices in browning callus derived from hypocotyls of *Jatropha curcas* was investigated. In this study, Yang investigated the effect of browning on callus morphology and biochemical indices. During browning, chlorophylls and carotenoid concentrations decreased steadily. Polyphenol oxidase (PPO) and peroxidase (POD) enzymatic activities pattern were similar during callus culture with a higher activity level at week 3 compared to week 2 or later weeks. Grey relation degree analysis indicated that PPO played a more important role than POD in enzymatic callus browning.

PPO may indirectly regulate endogenous IAA level in plant, through its action on phenolic substances. The catechol oxidase can occur in both latent and active forms in plant tissues. This enzyme has been reported from a variety of plant organs and tissues viz. pollen grains, latex, crown gall tissues and guard cells, respectively. This enzyme may be involved in some aspects of oxygen chemistry, perhaps mediation of pseudo cyclic photo-phosphorylation.

In the present studies PPO activity is negligible in *in vitro* plants as compared to *in vivo* plants. Similarly, various researchers have studied phenolic contents and PPO activity in diverse plant species *in vivo* and *in vitro* viz. *Cordia gharaf* and *Jatropha curcas* [21]; *Morus alba*, *Psoralea corylifolia* and *Boerhaavia diffusa* [22].

Indole acetic acid (IAA) oxidase has been reported to be involved in plant growth because of its alleged role in the control of endogenous IAA levels. Experiments indicate that approximately 70% of the IAA activity is at the cut surfaces of the tissue. In addition, up to 50% of the IAA oxidase activity could be pelleted in a membranous fraction, when the released enzyme was centrifuged at 10,000 rpm at 4°C for 10 minutes. Higher centrifugal forces reduced the proportion of the enzyme in the pellet, suggesting that vesicles containing IAA oxidase rupture at this force. However, sub cellular localization of IAA oxidase was accomplished by the use of sucrose density gradient centrifugation and fractionation.

It has been observed by Joseph et al. that the enzyme is associated most closely with Golgi complex and also to a lesser degree with the lysosomes and endoplasmic reticulum [23]. It has been suggested that IAA-oxidase is freely soluble in the cytoplasm and is therefore unlikely to have a role in controlling normal growth via IAA levels. The enzyme may have a role in diseased or damaged tissues.

The growth and IAA-oxidase activity of light-grown cucumber seedlings (cv. Aonagajibae) were investigated by Masayuki and Hiroshi in response to GA3 and IAA. Both GA3 and IAA induced significant elongation of the hypocotyls [24]. Treatment with GA3 or IAA resulted in retardation of IAA-oxidase activity in the hypocotyls and cotyledons. The degree of retardation was less in the cotyledons than in the hypocotyl. An inverse relationship was recognized between GA3 or IAA-induced elongation and IAA-oxidase activity in the hypocotyl.

Ascorbic acid or vitamin 'C' is also known as anti-scorbutic vitamin because of its curing action against scurvy. Ascorbic acid is a normal cellular constituent and its biosynthesis is considered to be an inherent property of actively growing plants [25]. The universal presence of ascorbic acid in actively metabolizing cells suggests that it has an important physiological role in plant growth and metabolism. Bharti reported changes in the activities of IAA oxidase, peroxidase and ascorbic acid utilization during BAP induced growth in cucumber cotyledons [26].

Ascorbic acid and its redox enzymes seem to be involved in the ageing processes and water stress condition. It is an important primary plant product and well known for its properties as an electron donor in photosynthetic photophosphorylation [27]. It is an important regulator of oxidation and plays a significant role in germination, growth and metabolism of flowering plants.

It stimulates amylase, protease, RNA activity and RNA content in various plants [28]. Considerable evidences are present in favour of its role in oxidative phosphorylation and its importance in animal health. In present study ascorbic oxidase activity has been higher in *in vitro* plant as compared to *in vivo* plant. Similarly, higher ascorbic oxidase activity has been reported by many workers [29,30]. Ascorbic acid has also been reported from wild fruits of Nigeria like *Sclerocarya birrea, Ximenia americana, Adansonia digitata, Zizyphus mauritiana, Balanites aegyptiaca, Tamarindus indica* etc. [31]. All actively growing and differentiating organs show higher concentration of ascorbic acid and it is constantly utilized enzymatically [28]. It is present in the cytoplasm and nucleus, both in bound as well as in free form. It induces resistance to drought and salinity in crops. Shimada et al. investigated the effects of ascorbic acid on Gingival melanin pigmentation *in vitro* and *in vivo* [32].

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