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Research Article

INTERACTION OF PEROXIDASES AND INDOLE-3-ACETIC ACID IN *ARMORACIA RUSTICANA*

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ABSTRACT
The naturally occuring auxin Indole-3-Acetic acid (IAA), is responsible for plant growth by cell elongation. Within plant cells, IAA concentration is controlled by the enzyme peroxidase. Peroxidases are also considered to be plant defence proteins due to their involvement in the lignification of cell walls, which restricts plant growth. The present study investigates whether exogenously supplied IAA can increase the production of peroxidase by *in vitro* plantlets of *Armoracia rusticana* or horseradish. Adding IAA to the growth medium was hypothesised to influence the activity of peroxidases in the horseradish plantlets, since IAA is a substrate of the enzyme peroxidase.
In the experiments, horseradish plantlets were grown in medium supplemented with increasing concentrations of IAA. The IAA treated plantlets showed a significantly higher growth rate than the controls. The growth rate of the treated plantlets increases linearly (r = +0.969) with the concentration of IAA in the medium up to 1.5 mg L^{-1} (0.105 mg d^{-1}) and was highest at this concentration. The plantlets grown with 0.5 mg L^{-1} IAA showed significantly higher peroxidase activity (0.516 U mL^{-1}) than the control plantlets (0.367 U mL^{-1}). The growth rate and the peroxidase activity share a negative correlation (r = –0.504) which may be due to the opposite functions of IAA and peroxidase in plants. Lower concentrations of IAA possibly mimic pathogen invasion, which enhances peroxidase activity to fortify the cell wall in response to the simulated pathogenic attack.
Our experimental results suggest that the peroxidase content of *in vitro* horseradish plantlets can be improved significantly using a low dose of IAA. This finding is important in tropical countries where horseradish, the commercial source of the multi-utility enzyme Horseradish peroxidase, does not grow naturally.

Keywords: *IAA, Auxin, Peroxidase, Armoracia Rusticana, Horseradish, Lignifications*

INTRODUCTION
Indole-3-Acetic Acid (IAA) is the best known naturally occurring auxin (Bryant and Lane, 1979). The role of IAA in plant growth and development is an important one. Several essential growth processes in plants, such as cell elongation, cell division, induction of root growth etc. involve IAA (Kukavica *et al.*, 2007). It is also used extensively in plant tissue culture (Dodd and Roberts, 1985; Nissen *et al.*, 1990). However, in order to streamline the growth process in a particular direction of development, the concentration of IAA in the target cells has to be regulated (Kukavica *et al.*, 2007). The concentration of IAA in target cells can be controlled by the action of the enzyme IAA-oxidase (Patel and Thaker, 2007), which is a type of plant peroxidase. Often, it has been found that plant peroxidases have IAA-oxidase activity, since an important function of these plant peroxidases is the catabolism of auxins (Cossio and Dunand, 2009).
The growth of plants by elongation is brought about by auxins like IAA, by altering the mechanical properties of the cell wall. This includes the enzyme mediated degradation of cell wall polysaccharides, which leads to loosening of the cell wall (Kotake *et al.*, 2000). Some plant pathogens like *Ralstonia solanacearum* that causes bacterial wilt in solanaceous plants induces a significant increase in IAA levels of infected plants. This increase in IAA levels increases the plasticity of the cell walls and makes the cell wall components like pectin, cellulose and protein more accessible and more liable to break-down by the degradative enzymes of the pathogen (Agrios, 2005).

The peroxidases have numerous roles in plants. Plant peroxidases may be cytoplasmic or cell wall associated (Meudt and Stecher, 1972; Vianello *et al.*, 1997). One of the primary roles of plant peroxidases is based on their ability to bring about the oxidative polymerization of aromatic monomers (Hollmann and Arends, 2012). This oxidative function is seen in the involvement of peroxidases in processes like lignification of the cell wall, suberin formation and the cross-linking of cell wall polymers (Veitch, 2004). Since peroxidases are involved with processes that fortify the plant cell walls, it is not surprising that peroxidases are also considered plant defense proteins that act against attacks from pathogens and insects (Cossio and Dunand, 2009). There are reports of exogenously applied IAA increasing the levels of cell wall associated peroxidase in tobacco (Meudt and Stecher, 1972; Henry *et al*., 1974), and pea plants (Kukavića *et al.*, 2007). This is probably an outcome of the interpretation of elevated IAA levels as a pathogenic attack, due to which the plant peroxidases act in order to strengthen the cell walls and control the spread of the pathogen.

The herb *Armoracia rusticana* or horseradish is native of temperate region. The plant does not set seed and is propagated vegetatively by means of its tuberous roots (Meyer and Milbrath, 1977). The horseradish plant is the commercial source of the enzyme horseradish peroxidase (HRP), which is a multi-utility oxidative enzyme. Since horseradish does not grow well in the tropics, micropropagation of this plant is required in the tropical regions. Improving the HRP content of the plant *in vitro* would be advantageous. In the present work, it was hypothesised that since IAA could be considered as a substrate for peroxidase, supplying IAA exogenously to the plant may cause an increase in the peroxidase content of the plant. Considering that IAA loosens the cell wall and plant peroxidases strengthen cell walls, the interaction of these two factors presented an interesting question. Also due to the fact that the plant horseradish was being micropropagated as a source of HRP, the addition of an optimum amount IAA to the growth medium is a relatively simple means of enhancing HRP production.

MATERIALS AND METHODS

Establishment of in vitro Culture

Plantlets of *Armoracia rusticana* were grown *in vitro* on modified Murashige and Skoog's (MS) medium (Dodd and Roberts, 1985) supplemented with 3% sucrose and gelled with 0.8% agar for 4-6 weeks. The nodes were used as explants. The plantlets were grown at an ambient temperature of 25±2 °C. The photoperiod selected was 8 hours light/ 16 hours dark. Light (photon density: 40.5 µmol m^{-2} s^{-1}) was provided using cool white fluorescent tube-lights.

Variation of IAA Concentration

After about 6 weeks, single, rooted plantlets weighing approximately 30 mg were selected and inoculated on modified MS medium supplemented with different concentrations of IAA (0.00, 0.25, 0.50, 1.00, 1.50 and 2.00 mg L^{-1}). The plantlets were grown under the same conditions of temperature and illumination as mentioned above.

Determination of Growth Rate

The plantlets were harvested after 3 weeks of growth and weighed. The growth rate of the plantlets grown with different concentrations of IAA was also calculated using the following formula (Birsin and Özgen, 2004)

Equation 1: $$RGR = \frac{(\text{Ln WF} - \text{Ln WI})}{T}$$

Where

RGR = Relative Growth Rate
W_I = Initial weight (mg)
W_F = Final weight (mg)
T = Growth period (days)

Preparation of Crude Peroxidase Extract

Approximately 0.2 g of shoot or root tissue of *A. rusticana* plantlets was weighed and macerated in a pre-chilled mortar and pestle with 2.0 mL of extraction buffer (pH 5.80 and 0.1 mol L^{-1}). The ratio between the weight of tissue (g) and the volume of buffer (mL) was maintained at 1:10. The macerated suspension was centrifuged at 3000 rpm for 5 min. The supernatant was used as the source of crude enzyme peroxidase.

Assay for Activity

The peroxidase activity of crude extracts of the entire plantlets was assayed using guaiacol as a substrate. The activity of peroxidase was estimated spectrophotometrically using a JASCO V-530 Spectrophotometer utilizing a method based on that of Kim and Yoo (1996). In the reaction, the coloured product tetraguaiacol is estimated at 470 nm (λmax).

The activity of an enzyme is expressed as units per mL (U mL^{-1}). One unit (U) is defined as the amount of enzyme that can convert 1 mole of substrate into product in one minute. Throughout the study, the activity of peroxidase in the crude extracts has been calculated using the formula given in Equation 2 (using guaiacol as substrate):

Equation 2: $$\frac{U}{mL} = \frac{\Delta OD}{min} \times \frac{RmV}{EV} \times \frac{dF}{\varepsilon_{470}}$$

ΔOD/min = increase in absorbance per minute (min^{-1})

ε_{470} = molar absorptivity of tetraguaiacol at 470 nm (mL μmol^{-1} cm^{-1})

RmV = reaction mixture volume (mL)

EV = enzyme extract volume

dF = dilution factor

RESULTS AND DISCUSSION

It was found during the course of the study that supplying IAA exogenously to the horseradish plants increased the growth rate of the plants significantly over the untreated controls. As seen in Figure 1, the growth rate increases linearly with the concentration of IAA, up to 1.5 mg L^{-1} (r = +0.969). Thereafter, it decreases as the concentration increases to 2.0 mg L^{-1}. The crude extracts of the plantlets grown with different concentrations of IAA, were tested for peroxidase activity towards guaiacol. It appears from Table 1 that 0.5 mg L^{-1} IAA is the optimum concentration for highest peroxidase activity (0.516 U mL^{-1}). Beyond this concentration, the peroxidase activity decreases significantly.

Table 1: Variation of growth rate and peroxidase activity with IAA concentration

Concentration of IAA (mg L^{-1})	Mean Growth Rate (mg d^{-1})	Mean Peroxidase activity (U mL^{-1})
0.00	0.049 ± 0.008^{c}	0.367 ± 0.011^{BC}
0.25	0.070 ± 0.015^{bc}	0.485 ± 0.009^{BC}
0.50	0.067 ± 0.007^{bc}	0.516 ± 0.038^{A}
1.00	0.084 ± 0.011^{ab}	0.372 ± 0.013^{BC}
1.50	0.105 ± 0.006^{a}	0.312 ± 0.070^{C}
2.00	0.085 ± 0.007^{ab}	0.293 ± 0.057^{C}

Note: Means (within a series) assigned the same letter(s) are not significantly different from each other at p=0.05

The trends of the growth rate as well as the peroxidase activity are shown in relation to the concentration of IAA in Figure 1. Both the factors share a moderate negative correlation (r=-0.504). The growth rate is a manifestation of the increased cell division and cell elongation (Bryant and Lane, 1979) under the influence of increasing concentrations of IAA. There are reports that suggest an inverse relationship between IAA levels and peroxidase activity in plants (Kukavica *et al.*, 2007). The inverse relationship may be explained based on the seemingly opposite functions of IAA and peroxidase. As mentioned before, IAA brings about growth in plants by elongation of cells and cell division. In the course of which, the plasticity of cell walls is increased due to the action of enzymes that specifically degrade cell wall

components (Agrios, 2005). On the other hand, the function of peroxidases is to improve the integrity of the cell wall by cross-linking cell wall polymers (Veitch, 2004).

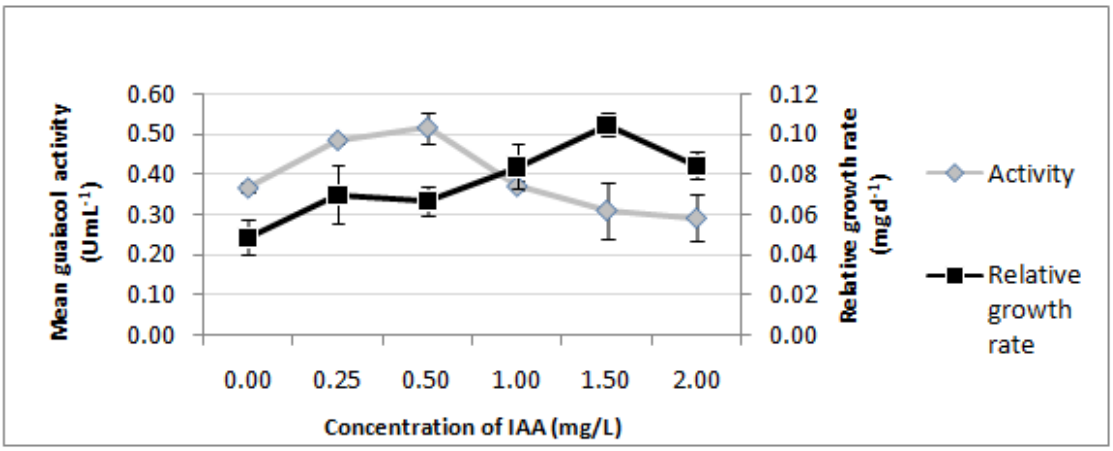

Figure 1: Effect of IAA on peroxidase activity and relative growth rate

However, Figure 1 shows that lower concentrations of IAA can increase the peroxidase content in the *A. rusticana* plantlets, due to which the crude extracts, show higher peroxidase activity. It appears as though lower concentrations of IAA simulate a situation akin to a pathogenic invasion. Many plants show slightly elevated IAA levels in the event of an attack by bacteria, fungi, viruses, nematodes and mollicutes (Agrios, 2005). However, beyond a certain concentration of IAA, 0.5mg L^{-1} in case of *A. rusticana*, the signal is interpreted by the plant as one of active growth. Therefore, the level of peroxidases that are likely to inhibit growth by stiffening the cell wall (Bacon *et al.*, 1997) is decreased. Hence, the Figure 1 shows that high growth rate and low peroxidase activity coincide beyond 0.5 mg L^{-1} IAA.

Conclusion

In the present study, the *A. rusticana* plantlets were grown *in vitro* with different concentrations of IAA. Further work is needed to determine which isoforms of peroxidases are inhibited and which ones are enhanced by IAA. The findings of the current study are significant in cases where *A. rusticana* plantlets are grown *in vitro* as a source of the enzyme horseradish peroxidase (HRP), since it is possible to use an optimum concentration of IAA to achieve a fairly high growth rate to provide adequate biomass and obtain the maximum peroxidase activity.

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