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Indolylacetic Acid Oxidase in Dormant Apple Embryos

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Abstract. The IAA oxidase activity was studied during the culture of dormant apple embryos. The effect of different factors on this enzyme activity was investigated either by adding them to the reaction mixture or to the culture medium. Phloridzin was found to be the best phenolic cofactor. The development of IAA oxidase activity was stimulated by phloridzin and GA_3. The properties of apple embryos IAA oxidase allow to postulate the presence of two enzyme systems able to oxidize IAA in the material studied. The involvement of peroxidase activity in IAA oxidation was also investigated. The differences in the changes of peroxidase and IAA oxidase activities during the culture of dormant apple embryos do not permit to consider the activity of peroxidases to be identical with that of IAA oxidase.

Studies of several authors *e.g.* NIKOLAEVA (1967), GORDON-SHAHIR and WAREING (1964) indicate the auxin superoptimal concentration to be one of the factors responsible for the maintaining of dormancy in some of the seeds.

The attempts to demonstrate the presence of auxin in dormant apple seeds have failed to give univocal results. The presence of three auxin-like substances in maturing seeds had been stated by LUCKWILL (1957) and in 1962, ethyl-3-indolylacetate and 3-indolylacetylaspartic acid were identified by RAUSSENDORF-BARGEN in the same material. No auxin accumulation was stated in the dormant apple seeds (LUCKWILL 1969).

The control of auxins concentration in the tissue depends upon their biosynthesis and degradation processes.

The present work was aimed at characterization of indolyl-3-acetic acid oxidase (IAA oxidase) in embryos from non-stratified apple seeds. Considering the postulated involvement of peroxidases in IAA-oxidase complex (GALSTON and DAVIES 1962, PARIS 1969, HARE 1964, BOLDUC *et al.* 1970) as well as the marked peroxidase activity in apple seeds (RYCHTER and LEWAK 1971) a correlation between the peroxidases and IAA oxidase activities was given a trial.

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Material and Methods

Material and Culture Conditions

Non-stratified seeds of apple tree (*Pyrus malus* L., cv. Antonówka) were used throughout our experiments. The isolated apple embryos were cultured for 10 days, in the presence of different solutions or water under the conditions described previously (SMOLEŃSKA and LEWAK 1971).

Following solutions were used:

1. 5×10^{-6} M indolyl-3-acetic acid (IAA)
2. 5×10^{-5} M gibberellin A_3 (GA_3)
3. 2×10^{-2} M tryptophane and 10^{-2} M chlorogenic acid
4. 2×10^{-2} M tryptophane and 10^{-2} M protocatechuic acid
5. 10^{-3} M phloridzin

Embryos for preparation of acetone powder were taken out at two days intervals. The acetone powders were prepared as described previously (RYCHTER and LEWAK 1969).

Extracts Preparation

Amounts of acetone powder corresponding to 60 seeds were extracted with 10 ml 0.02 M phosphate buffer, pH 6.2. The extraction was carried out for 1 h at 4 °C. IAA oxidase and peroxidase activities were assayed on the same day.

IAA Oxidase Activity Assay

0.4 ml of enzyme extract and 0.5 ml of 0.2 M phosphate buffer (100 μmoles), pH 6.2 were added to 2.5 ml of reaction mixture consisting of 3.75×10^{-7} moles IAA, 2.5×10^{-7} moles $MnCl_2$, and 2.5×10^{-7} moles phenolic cofactor. Reference sample was prepared in the same way, the enzyme extract being added after heat denaturation for 15 min in boiling water bath. The reaction was carried out for 30 min then stopped by adding 5.0 ml of Salkowski reagent. The absorbance was measured after another 30 min at 530 nm using Specol spectrocolorimeter.

The IAA oxidase activity was expressed as the μmoles IAA destroyed within 1 h by the enzyme extract obtained from 100 seeds.

Peroxidase Activity Assay

0.1 ml of enzyme extract was added to 6.1 ml of reaction mixture containing 4 μmoles of benzidine, 200 μmoles of acetate buffer, pH 5.0 and 100 μmoles of H_2O_2. The absorbance was measured after 30 s at 590 nm.

The peroxidase activity was expressed as ΔA at 590 nm per 100 seeds.

Results

The IAA oxidase activity assays in embryos grown for 6 days, were performed using the different phenolics as cofactors. (Table 1.) Phloridzin was proved to be the most effective cofactor as its presence in the incubation mixture results in a 3.5 fold rise of IAA oxidase activity. The presence of quercetin resulted in 50% rise of IAA oxidase activity and complete inhibition of the enzyme activity was observed with chlorogenic acid. The addition of both phloridzin and chlorogenic acid resulted only in the inhibition of IAA oxidation. Attention is called to the fact that phloridzin and quercetin as well as chlorogenic acid are the native phenolics of the apple seeds. The exogenous ones *i.e.* 2,4-dichloro-phenol and ferulic acid are of no effect on the IAA oxidase activity.

The IAA oxidase activity was investigated in embryos soaked for 24 h in water and during the 10 days water culture. The enzyme activity was assayed parallelly in the incubation mixture not containing any phenolic cofactor and in the presence of phloridzin.

TABLE 1

IAA OXIDASE ACTIVITY IN NON-STRATIFIED APPLE EMBRYOS CULTURED FOR 6 DAYS, MEASURED IN THE PRESENCE OF DIFFERENT PHENOLIC COMPOUNDS AS COFACTORS

Phenolic cofactor (conc. in incubation mixture)	IAA oxidase activity	
	IAA destroyed [μmol h^{-1} per 100 embryos]	[%]
without cofactor	5.9	100
phloridzin 7.35×10^{-5} M	21.0	356
quercetin 3.67×10^{-5} M	9.22	156
chlorogenic acid		
7.35×10^{-6} M	0	0
7.35×10^{-5} M	0	0
phloridzin + chlorogenic acid		
7.35×10^{-5} M + 7.35×10^{-6} M	0	0
7.35×10^{-5} M + 7.35×10^{-5} M	0	0
2,4-dichlorophenol 7.35×10^{-5} M	6.0	102
ferulic acid 7.35×10^{-5} M	5.33	90.5

The results presented in Fig. 1 indicate that phloridzin is of no effect on the activity of IAA oxidase present in the extracts of embryos isolated from non-stratified apple seeds. During the embryos culture the phloridzin dependent IAA oxidase activity appears. The IAA oxidase activity manifesting without any phenolic cofactor remains unchanged during the embryo culture.

A rapid rise in the IAA oxidase activity, measured in the presence of phloridzin, is noted between the 2nd and the 4th day of culture. This maximum persists until the 6th day, then gradually decreases and only 50% of maximum activity is noted on the 10th day of culture. As phloridzin had been stated the most active IAA oxidase stimulating cofactor of the phenolics studied, the relation between phloridzin concentration and IAA oxidase activity was investigated.

The results are presented in Table 2. The highest IAA oxidase activity corresponded to the phloridzin : IAA ratio 1 : 1 *i.e.* to the respective 7.35×10^{-5} to 1.47×10^{-4} M concentrations in the incubation mixture.

TABLE 2

INFLUENCE OF PHLORIDZIN CONCENTRATION ON THE IAA OXIDASE ACTIVITY. ENZYME EXTRACTS WERE PREPARED FROM DORMANT APPLE EMBRYOS CULTURED FOR 6 DAYS. IAA CONCENTRATION IN REACTION MIXTURE — 1.1×10^{-4} M.

Phloridzin concentration in incubation mixture	IAA oxidase activity IAA destroyed [μmol h^{-1} per 100 embr.]
7.35×10^{-6} M	6.3
7.35×10^{-5} M	18.9
1.47×10^{-4} M	20.1
3.67×10^{-4} M	15.2

Relation between pH of the incubation mixture and the IAA oxidase activity with phloridzin as a cofactor is presented in Fig. 2. Two maxima — one at pH 4.0 and the other at pH 5.8—6.2 are observed.

Further assays of IAA oxidase activity were performed in phosphate buffer pH 6.2 as the enzyme is more stable in neutral medium.

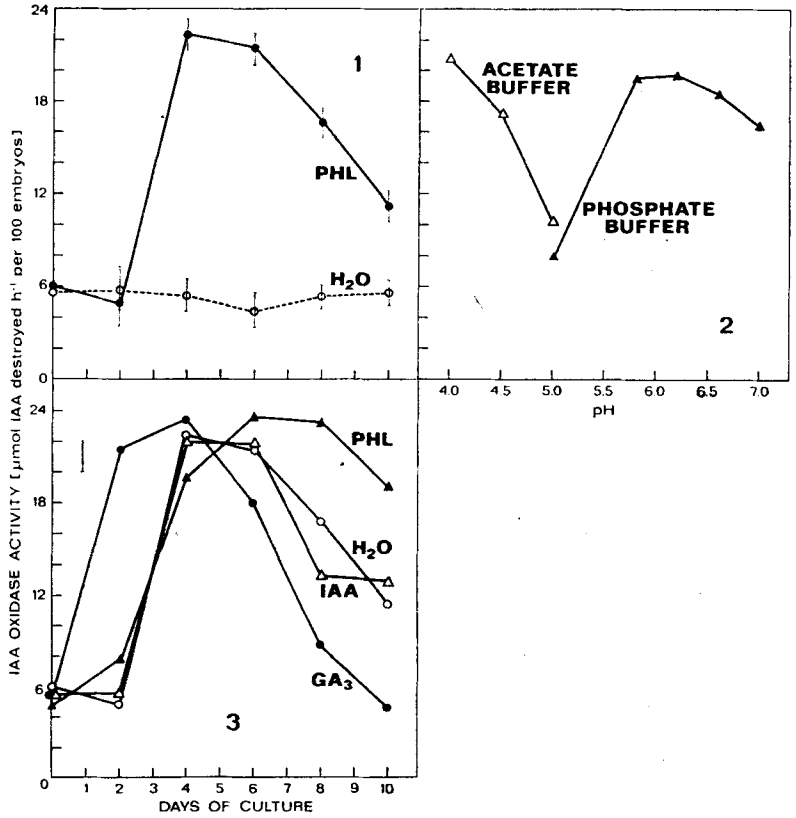

Fig. 1. Changes in IAA oxidase activity in apple embryos, during their culture on the water, measured without phenolic cofactor (H_2O) or in the presence of phloridzin (PHL).
Fig. 2. pH-activity curve for oxidation of IAA by enzymatic extract from apple embryos cultured for 6 days.
Fig. 3. Changes in IAA oxidase activity in apple embryos cultured in the presence of phloridzin (PHL), IAA, GA_3, and of water as control. The activity was measured with phloridzin as phenolic cofactor.

Because of the postulated inductive character of the IAA oxidase (LEE 1971, STUBER and LEWINGS 1969) experiments were carried out with isolated embryos grown for 10 days in the presence of IAA, GA_3 or phloridzin. Changes in the IAA oxidase activity in those embryos are presented in Fig. 3.

The presence of IAA in the culture medium does not affect the IAA oxidase activity during the 10 days of culture and the pattern of enzyme activity

is similar to that of the control embryos grown on water (Fig. 1). The presence of GA_3 accelerates the appearance of IAA oxidase activity without affecting its value. The rapid rise in enzyme activity was observed between the beginning and the 2nd day of culture. This maximum persists until the 4th day of culture and then the IAA oxidase activity decreases.

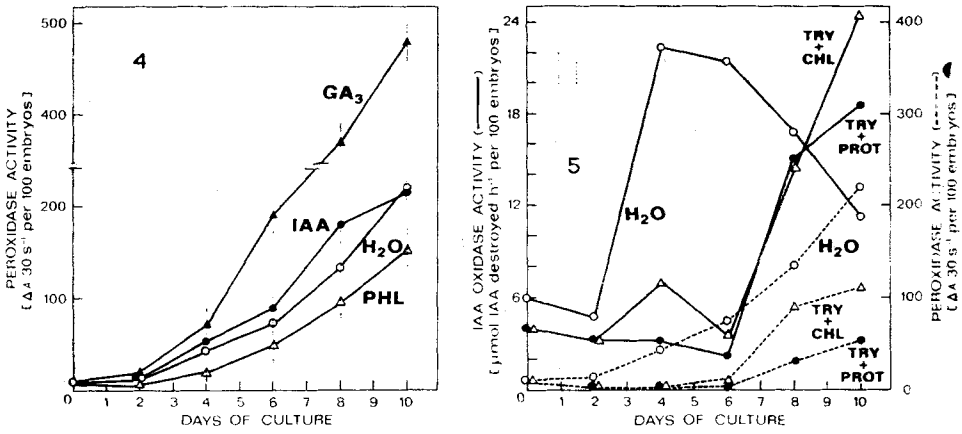

Fig. 4. Changes in the peroxidase activity in apple embryos, cultured in the presence of phloridzin (PHL), IAA, GA_3 and of water as control.
Fig. 5. Changes in the IAA oxidase and peroxidase activities in apple embryos cultured on the solution of tryptophane with chlorogenic acid (TRY + CHL) or tryptophane with protocatechuic acid (TRY + PROT) and with water as control.

In extracts from embryos cultured on phloridzin solution, the IAA oxidase activity reaches its maximum on the 6th day with a plateau lasting till the 8th and a subsequently slight decrease between the 8th and 10th day.

Peroxidase activity was also assayed in the same extracts and the results are presented in Fig. 4. Attention should be drawn to the differences between the dynamics of peroxidase and IAA oxidase activities. The activity of peroxidases rises throughout the whole 10 days period of culture, whereas the IAA oxidase activity decreases after reaching its maximum. If the peroxidases activity in extracts from embryos cultured on water is accepted as a reference value, this activity is strongly stimulated by GA_3 (over 100%) throughout the whole period of culture. IAA is of slight stimulating effect only and phloridzin causes a 30% decrease of activity as compared to water control.

The data presented in Fig. 3 proved that the IAA oxidase in apple embryos is not an enzyme induced by IAA. Taking into account that tryptophane has been commonly postulated to be the precursor of auxins (KUTÁČEK and KEFELI 1968) and the involvement of *o*-diphenols in the auxins biosynthesis has been suggested by GORDON and PALEG (1961) an attempt was made at checking whether IAA oxidase is induced by any of the metabolites preceding IAA in the process of biosynthesis. The determinations of IAA oxidase and peroxidase activities were performed in extracts from embryos cultured for 10 days on solutions containing tryptophane with chlorogenic acid or

tryptophane with protocatechuic acid. The results are presented in Fig. 5. Both enzyme activities remain unchanged during the first 6 days of embryo culture. Their subsequent rise leads, in the case of IAA oxidase activity, to the values higher than that of the control, but peroxidase activity remains inhibited by tryptophane with *o*-diphenols. It is difficult to interpret these results as an induction of the studied enzymes.

Discussion

As it was already shown (DZIEWANOWSKA *et al.* 1974), the IAA oxidase activity which appears during maturing of the apple seeds is independent of the cofactor used.

Our results suggest the existence of two kinds of enzyme systems which are able to oxidize IAA. One of them, present in the dormant embryos (possibly the same system which is active in the maturing seeds) does not need any phenolic cofactor and its activity remains unchanged during the embryo culture. Another IAA oxidase system, of more complicated oxidation mechanism susceptible to exogenous factors and needing the presence of phenolic cofactor appears during the culture. The addition of phloridzin to the incubation mixture is of optimal effect if the phloridzin: IAA ratio is 1 : 1. The increase in phloridzin concentration alone results in the decrease of IAA oxidase activity may be by blocking of the IAA binding site. Phloridzin which has been proved to be the most effective IAA oxidase cofactor in the apple embryos, appears in the embryos since the 4th day of water culture (BOGATEK *et al.* — in preparation). Its appearance correlates with the manifestation of IAA oxidase activity. The presence of gibberellin in the cultured medium accelerates the start of phloridzin biosynthesis; the same effect was observed for the appearance of IAA oxidase activity. Thus the accelerating effect of gibberellin on the appearance of this enzyme activity may be explained by the accelerating effect of the hormone on phloridzin biosynthesis which is indispensable for the IAA oxidase activity.

The differences in the dynamics observed between the peroxidases and IAA oxidase activities do not allow to consider those two enzymes to be identical. The peroxidase activity rises throughout the whole period of culture, while that of IAA oxidase decreases after reaching its maximum. The presence of phloridzin in the culture medium results in a rise in the IAA oxidase activity and prevents its disappearance whereas it causes 30% inhibition of peroxidases activity.

Taking into account the heterogeneity of apple embryos peroxidases which is manifested by differences in electrophoretical mobility as well as in substrate specificity (RYCHTER and LEWAK 1971), the involvement of one of isoperoxidases in the IAA oxidase activity may be accepted.

Suggestions concerning the physiological role of IAA oxidase in the apple seeds may be drawn first after the assay of auxins content in the material studied.

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K. DZIEWANOWSKA, S. LEWAK (Warszawa): **Oxidáza kyseliny indolyloctové v dormantních jabloňových embryích.** — Biol. Plant. **17** : 207—213, 1975.

Byla sledována aktivita oxidázy IAA během pěstování dormantních jabloňových embryí. Vliv různých faktorů na aktivitu tohoto enzymu byl sledován buď jejich přidáním do reakční směsi nebo do kultivačního media. Bylo zjištěno, že floridzin je nejlepší fenolický kofaktor. Aktivita oxidázy kyseliny indolyloctové byla stimulována floridzinem a GA_3. Vlastnosti IAA oxidázy jabloňových embryí prozrazují přítomnost dvou enzymatických systémů schopných oxidovat kyselinu indolyloctovou ve studovaném materiálu. Dále byla sledována závislost aktivity peroxidázy na oxidaci IAA. Rozdíly ve změnách aktivity peroxidázy a IAA oxidázy během kultivace dormantních jabloňových embryí nesvědčí pro identitu aktivity peroxidázy a IAA oxidázy.