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# Early changes of peroxidase activity and of endogenous free auxin level in micropropagated lupin shoots transferred on a rooting medium

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ABSTRACT

JOUVE, L., I. CLARIS-HARDY, L.-M. DUPUIS & Th. GASPAR (1994). Early changes of peroxidase activity and of endogenous free auxin level in micropropagated lupin shoots transferred on a rooting medium. *Saussurea* 25: 75-81. In English, English and French abstracts.

Changes in peroxidase activity and in level of free endogenous IAA have been investigated in lupin shoots on a root induction medium (with exogenous auxin), in relation with the rooting performance after transfer on a root expression medium (without auxin). Rooting of lupin shoots needed a passage of at least 10 hours on the auxin-based medium to form healthy roots on the expressive medium. Longer time-cultures on the first rooting medium increased rooting performance (percentage, laterals). Peroxidase activity showed a rapid decrease up to a minimum in the 9 first hours culture on the root induction medium. In the same time, an inverse variation was shown in free IAA level with a peak occurring after 6 hours culture on the first rooting medium. Then, peroxidase activity increased slowly up to a maximum after 42 hours culture while IAA level fluctuated at relatively low levels. This indicated that peroxidase activity or free auxin level could be good indicators of root formation.

RÉSUMÉ

JOUVE L., I. CLARIS-HARDY, L.-M. DUPUIS & Th. GASPAR (1994). Modifications précoces de l'activité peroxydasique et du contenu en auxine endogène libre de pousses micropropagées de lupin sur milieu d'enracinement. *Saussurea* 25: 75-81. En anglais, résumés en anglais et français.

Les modifications de l'activité peroxydasique et du contenu endogène en IAA libre de pousses micropropagées de lupin sur un milieu inductif d'enracinement (avec auxine exogène) ont été mesurées et mises en relation avec le taux d'enracinement ultérieur sur un milieu d'expression racinaire (sans auxine). L'enracinement des pousses de lupin requiert un passage d'un minimum de 10 heures sur le milieu auxinique pour former des racines fonctionnelles sur le milieu d'expression. Des temps de passage plus longs sur le milieu auxinique augmentent le taux d'enracinement et la production de racines latérales. L'activité peroxydasique diminue rapidement jusqu'à un minimum dans les 9 premières heures de culture sur milieu d'induction. Le niveau en IAA endogène subit une variation inverse avec un pic après 6 heures de culture. L'activité peroxydasique s'accroît ensuite progressivement jusqu'à un maximum après 42 heures tandis que le taux en IAA fluctue à des niveaux relativement bas. L'activité peroxydasique autant que le taux en IAA sont donc de bons indicateurs du déroulement du processus d'enracinement.

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## Introduction

Rooting of micropropagated shoots does not necessarily issue as a result of their transfer on an auxin-based rooting medium (DAVIS & al., 1988; DAVIS & HAISSIG, 1994). It may need the use of several successive media (MONCOUSIN & GASPAR, 1983; BERTHON & al., 1990; RIPETTI & al., 1994), but the establishment of such media still remains empirical. This is the reason why investigations have been programmed to evidence biochemical markers of the time-course progression of the rooting process (JARVIS, 1986; MONCOUSIN, 1991). Although endogenous auxins were recognized to play a central role in this developmental phenomenon, this role remained unclear (GASPAR & HOFINGER, 1988; BLAKESLEY & al., 1991). It seems to be somehow clarified recently (BLAKESLEY, 1994; GASPAR & al., 1994) due to two types of observations made from several sides. First, root formation has been shown to occur as a series of interdependent phases and is no longer considered as a single physiological process (MITSUHASHI-KATO & al., 1978; MONCOUSIN & al., 1989). Second, the systematic study of peroxidase activity in the course of rooting has led to decompose the process in at least three well marked phases (GASPAR & al., 1992, 1994): an inductive phase achieved through a rapid decline of peroxidase activity up to a minimum, a following initiative phase corresponding to an increase of peroxidase activity up to a peak, and an expressive phase characterised by a progressive decline of enzyme activity. The level of endogenous free auxin follows an inverse pattern in the course of the three phases (MONCOUSIN & al., 1989; GASPAR & al., 1994). The data indicate that the initiative phase of rooting, corresponding to a temporary decline of peroxidase activity and temporary peak of auxin level, may occur very rapidly after the shoots transfer from the multiplication to the rooting medium, even at the end of the multiplication cycle, which may explain that these changes had not been recorded in anterior investigations. This also explains the discrepancies in the results of auxin titration in the course of rooting studies (DAVIS & al., 1988; DAVIS and HAISSIG, 1994).

We are actually confronted with the difficulties in the rooting of micropropagated shoots of several lupin species, and are looking to improve our cultures using the above markers. The changes of peroxidase activity and of the level of endogenous free auxin have been here determined in shoots of Lupinus mutabilis where the first rooting successes were obtained (HARDY & al., 1994). It is hoped to exploit the reported results in order to achieve and (or) perform rooting in other lupin species and varieties.

## Material and methods

### *Material and culture*

An *in vitro* proliferating shoot culture of *Lupinus mutabilis* was initiated from cotyledonary axillary buds of seedlings raised *in vitro* (HARDY & al., 1994). Maintenance of the proliferating cluster used a MURASHIGE & SKOOG (1962) medium (MS) with 3 $mg.l^{-1}$ BAP, in a growth culture room (24°C, 16 h photoperiod, light intensity of 2.9 $W.m^{-2}$ provided by Sylvania Gro-Lux F30W fluorescent lamps) through subcultures every 4 weeks. 5 to 10 mm long (1 to 2 nodes) cuttings from such cultures transferred on a classical rooting medium (several auxins at varying concentrations were tested) did not root. They needed a prior passage on an elongation medium (MS with 2 $g.l^{-1}$ active charcoal, without regulators) for 3 weeks. Afterwards microcuttings (30 to 50 mm long; 3 to

5 nodes) were placed on a first rooting medium (MS supplemented with 3 $mg.l^{-1}$ IBA) for root induction and initiation for a variable time, then on a second rooting medium (composition in Table 1, no growth regulators) for 4 weeks for rooting expression.

All media were supplemented with 30 $g.l^{-1}$ sucrose and adjusted to pH 5.8 with KOH or HCl prior to the addition of Roland (Brussels) agar (8 $g.l^{-1}$) and subsequent autoclave sterilization for 20 min at 121°C and 118 kPa. Multiplication and elongation cultures (8 shoots per container) were grown on 80 ml medium in 300 ml rectangular (10.8 × 8.2 cm; 5.5 cm height) plastic containers, with plastic adjustable lids (from Reynolds Film Inc.). Rooting cultures (8 shoots per jar) were grown on 80 ml medium in 375 ml cylinder (8.5 cm diameter) "Meli" glass jars closed with polypropylene screw lids.

*Peroxidase activity and auxin level*

Lupin shoots from the inductive rooting medium were collected every 3 hours during 51 hours for measurement of their soluble peroxidase activity. Frozen shoots (150 mg of fresh weight) were ground with cold mortar and pestle in 1.5 ml phosphate buffer 0.06 M, pH 6.1, supplemented with solid polyvinylpyrolidone (Polyclar w/w) and centrifuged for 10 min at 10,000 *g* at 4°C. The peroxidase assays were performed as previously reported (MONCOUSIN & GASPAR, 1983). The activity was expressed in µg equivalent commercial HRP (horseradish peroxidase from Fluka) per mg protein. Protein concentration was determined by the Coomassie blue method (SPECTOR, 1978). The results are the mean of five separate experiments.

The extraction and determination of free IAA in the shoot samples collected during the first 33 hours from the inductive rooting medium were done according to the technique of NORDSTRÖM & al. (1991), already practiced in the laboratory (HAUSMAN, 1993). The level of IAA was expressed in ng per g fresh mass. The results are the mean of three separate experiments.

## Results

*Rooting rate*

The rooting percentages counted on the second rooting medium in relation to the duration of the passage of the first auxin-based rooting medium are shown in Table 2. There was 30% rooting when the shoots did not pass on the first medium, but the roots appeared very fragile and did not develop laterals. 40% and 60% of the shoots which had been cultured 10 and 20 hours respectively on the first medium formed healthy roots without laterals. 70 to 80% of the shoots produced adventitious roots with passage times from 30 to 50 hours on the first rooting medium: the roots were longer than these formed with shorter passage times and developed laterals.

*Peroxidase activity and IAA level*

Changes of peroxidase activity of the lupin shoots during a 51 h passage on the auxin-based first rooting medium are shown in Figure 1. A rapid decrease of peroxidase activity up to a minimum occurred in the 9 first hours. Peroxidase activity reincreased afterwards with several maxima culminating at the 42th hour, before a considerable decrease.

The time-course variation of the free IAA level is shown in Figure 2. A sharp temporary peaking occurred between the 3th and 9th h, thus showing an inverse variation of that of peroxidase activity. The IAA level then fluctuated at relatively low levels while peroxidase activity was at high levels.

Table 1. — Composition of the expressive (second) rooting medium.

| | *mg.l-¹* | *mM* |
|---|---|---|
| $NH_4NO_3$ | 50 | 0.6247 |
| $KNO_3$ | 1650 | 16.3205 |
| $Ca(NO_3)_2$, 4 $H_2O$ | 499.6 | 2.1156 |
| $MgSO_4$,7 $H_2O$ | 1350 | 5.4771 |
| KCl | 100 | 1.3414 |
| $Na_2SO_4$ | 44 | 0.3098 |
| $KH_2PO_4$ | 120 | 0.8818 |
| | 1 | |
| $MnSO_4$, $H_2O$ | 1.692 | $1.10^{-2}$ |
| $H_3BO_3$ | 0.62 | $1.10^{-2}$ |
| $ZnSO_4$, 7 $H_2O$ | 0.86 | $3.10^{-3}$ |
| $Na_2MoO_4$, 2 $H_2O$ | 0.0215 | $8.89.10^{-3}$ |
| $CuSO_4$, 5 $H_2O$ | 0.0025 | $1.10^{-5}$ |
| $CoCl_2$, 6 $H_2O$ | 0.0025 | $1.05.10^{-5}$ |
| Meso-Inositol | 100 | 0.5551 |
| Pyridoxine, HCl | 0.5 | $2.43.10^{-3}$ |
| Nicotinic acic | 0.5 | $4.06.10^{-3}$ |
| Calcium Penthotenate | 0.5 | $1.05.10^{-3}$ |
| Biotine | 0.01 | $4.09.10^{-5}$ |
| Glycine | 2 | $2.66.10^{-2}$ |
| Fe-EDTA | 100 | 0.263 |
| Active charcoal | 2500 | |

Table 2. — Rooting of lupin shoots after several durations on the first rooting medium and 4 weeks on the second one.

| *Hours on first rooting medium* | *Rooting (%)* | *Root length (mm)* | *Secondary roots (yes / no)* |
|---|---|---|---|
| 0 | 30 | 5- 20 | no |
| 10 | 40 | 20- 45 | no |
| 20 | 60 | 30- 60 | no |
| 30 | 70 | 40-100 | yes |
| 40 | 70 | 40-100 | yes |
| 50 | 80 | 40-100 | yes |

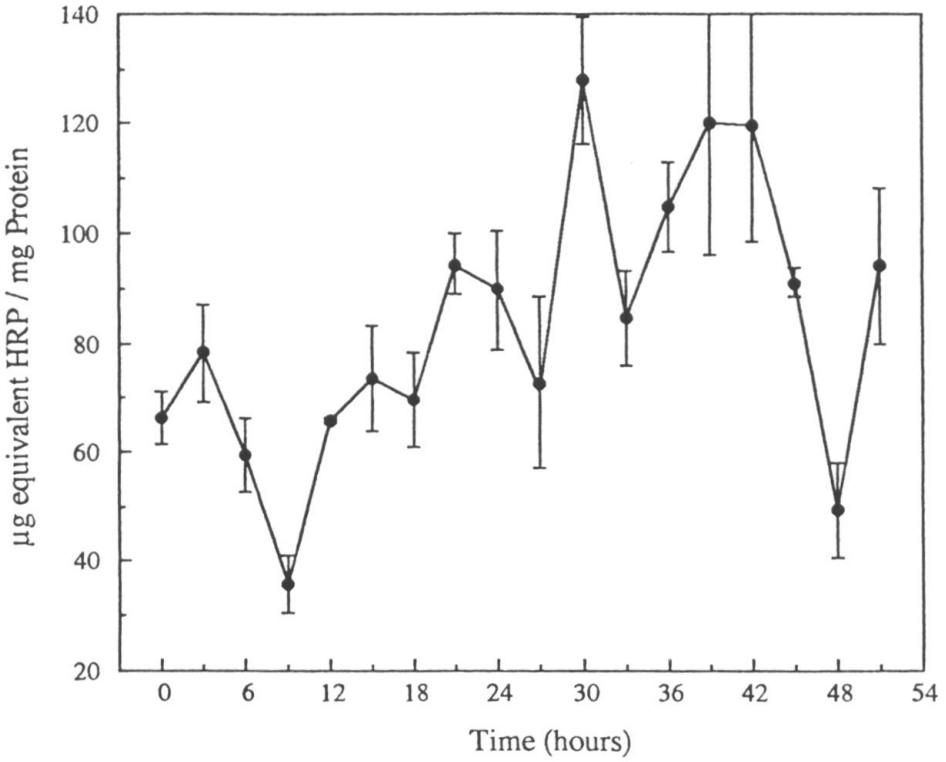


Fig. 1. — Changes in peroxidase activity in lupin shoots cultured on the first rooting medium (3 mg.l$^{-1}$ IBA).

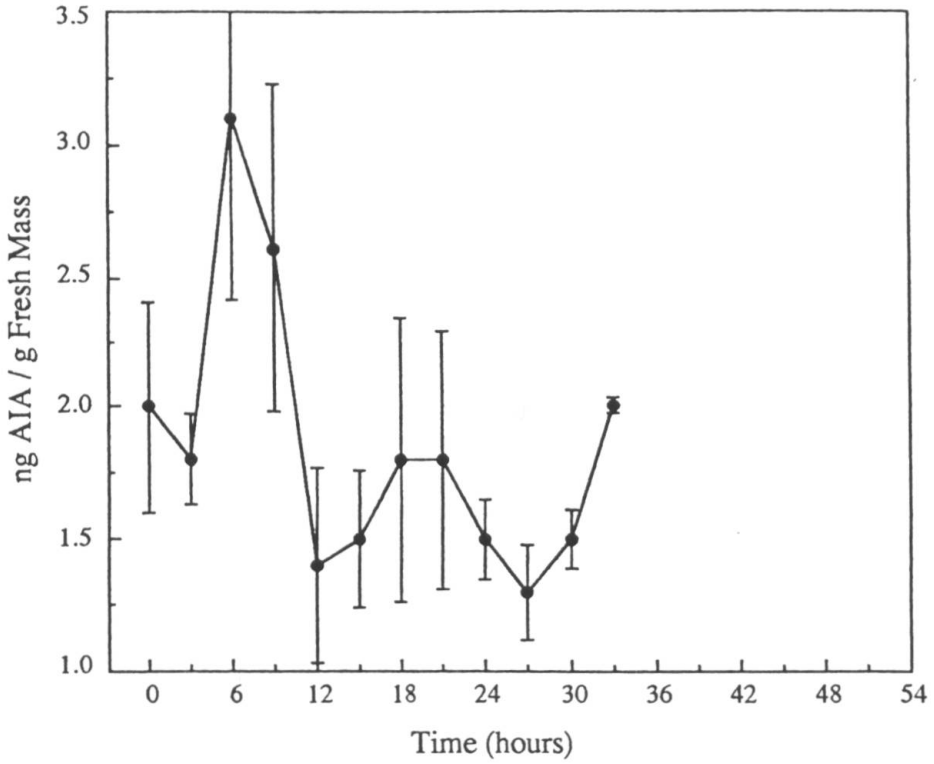


Fig. 2. — Changes in IAA content in lupin shoots cultured on the first rooting medium (3 mg.l$^{-1}$ IBA).

## Discussion

The changes of peroxidase activity here determined, with an early minimum followed by a considerable although slower increase and peaking, were identical to those already observed in the course of rooting in vine cuttings (MONCOUSIN & al., 1989), in poplar shoots (HAUSMAN, 1993) and walnut shoots (RIPETTI & al., 1994). The inverse variation of the free auxin levels also well corresponded to those mesured in vine and poplar cuttings (MONCOUSIN & al., 1989; HAUSMAN, 1993). According to BLAKESLEY (1994) and GASPAR & al. (1994), the early peroxidase minimum or the early auxin peak marked the termination of the root induction phase, before any cytological event. The further peroxidase increase and the maintenance of low IAA levels could correspond to the initiative phase of rooting, i.e. the cell divisions forming internal morphogenic cell clusters in which meristemoids and root primordia will be organised, starting so the so-called expressive rooting phase. Early studies by DRUART & al. (1982), MONCOUSIN & GASPAR (1983) and BERTHON & al. (1993) have shown that influencing peroxidase activity from the exterior by physical or chemical factors might beneficially improve the rooting rate. The external and internal factors favouring the completion of the successive rooting phases in lupin shoots are being investigated with the aim of getting rooting in the up-to-now recalcitrant lupin species and (or) varieties. The present study seems to indicate that both the inductive and initiative phases of lupin rooting required the presence of an exogenous auxin while the expressive phase did not. The causal relationship between the changes of peroxidase activity and free auxin level will also be investigated with the same purposes.

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