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Hormones and Pod Development in Oilseed Rape (*Brassica napus*)[1]

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ABSTRACT

The endogenous levels of several plant growth substances (indole acetic acid, IAA; abscisic acid, ABA; zeatin, Z; zeatin riboside, [9R]Z; isopentenyladenine, iP; and isopentenyladenosine, [9R]iP were measured during pod development of field grown oilseed Rape (*Brassica napus* L. var *oleifera* cv Bienvenu) with high performance liquid chromatography and immunoenzymic (enzyme-linked immunosorbent assay, ELISA) techniques. Results show that pod development is characterized by high levels of Z and [9R]Z in 3 day old fruits and of IAA on the fourth day. During pod maturation, initially a significant increase of IAA and cytokinins was observed, followed by a progressive rise of ABA levels and a concomitant decline of IAA and cytokinin (except iP) levels. The relationship between hormone levels and development, especially pod number, seed number per pod, and seed weight determination, will be discussed.

Brassica napus is one of the main oil and protein producing plants grown in Europe; therefore, its culture is of the utmost importance. Yield components, pod number, seed number per pod, and seed specific weight do not only depend on nutritional factors but also on hormonal ones. Morgan (16) showed that seed number per pod could be improved with a 4-chlorophenoxyacetic acid application 15 d postanthesis. Later, Inanaga and Kumura (11) provided evidence that seed number per pod was mainly correlated to IAA levels during the early days of development. Crosby *et al.* (7) and Carlson *et al.* (5) succeeded in raising the pod number in *Glycine max* plants with 6-benzylaminopurine treatments. Morgan *et al.* (17), who experimented with BA treatments on oilseed rape, succeeded in improving flower number, although they failed to increase pod number. Soybean seed growth was correlated to ABA levels (21). ABA treatments enhance sucrose uptake by soybean embryos (22). ABA may also be involved in protein synthesis and accumulation in seeds (8, 9). In fact, partitioning of assimilates could be under ABA control (3). Although the involvement of PGRs[2] in pod development seems undeniable, very little is known on the dynamics of the endogenous hormone status. Therefore, with the goal of improving understanding on relationships between PGRs and pod development, we measured endogenous levels of IAA, ABA, Z, [9R]Z, iP, and [9R]iP in pods of field grown oilseed rape plants from 1 to 47 d postanthesis.

MATERIALS AND METHODS

Plant Culture

Winter rape (*Brassica napus* L. var *oleifera*, cv Bienvenu) were sown on September 5, 1986, in field (Research Station, Institut National Agronomique Paris-Grignon, Grignon, France) with normal agronomic techniques in deep (1 m) alluvium (40%) soil with satisfactory mineral composition (0.025% K_2O, 0.030% P_2O_5, 3% $CaCO_3$) and satisfactory water retention capacity. Rainfall and temperature were measured daily (Station de Bioclimatologie, Grignon, France).

Sampling

Most of the pods are produced on the terminal inflorescence, and final yield is composed essentially of seeds formed during the first week of flowering. Moreover, pod development during this period is more regular (6), while most flower and pod abortion takes place early after flower opening. Therefore, all pods were sampled at beginning of flowering from the terminal inflorescences of 30 plants in order to have significant mean values and negligible interplant variation. On each plant, all pods were taken at a precise age counted from anthesis. Sampling was spaced regularly from 1 to 47 d, but with a special attention focused on the first week development (1 sample/d). After excision, organs were frozen in liquid N_2, lyophilized, and ground to powder before storage. Sampling always occurred at the same hour, in order to limit diurnal variation of hormonal levels, especially of ABA (25). Sample characteristics are given in Table I.

Extraction and Purification of Samples

The methods employed have already been reported (13, 15, 23) and therefore will be only briefly described. Twenty mg samples were homogenized in 5 mL 80:20 (v/v) methanol:distilled water containing 40 mL/L butylhydroxytoluene as an antioxidant. Radiolabeled hormones ([^{3}H] *cis* (±) ABA, specific activity 63.6×10^{10} Bq/mmol from the Radiochemical Centre, Amersham, UK; [5(n)-^{3}H]IAA, specific activity

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[2] Abbreviations: PGR, plant growth substance; Z, zeatin; (9R)Z, zeatin riboside; iP, isopentenyladenine; (9R)iP isopentenyladenosine; DW, dry weight.

Table I. *Characteristics of the Material at Sampling Time*

Dates 1987	Ages	Temp.[a]	Length
	d		*mm*
4–26	1	14	5
4–27	2	31	7
4–28	3	45	10
4–29	4	60	15
4–30	5	80	18
5–1	6	94	24
5–2	7	111	28
5–15	19	237	76
5–22	26	295	80
5–31	35	416	80
6–12	47	583	78

[a] Mean day temperature (>0°C) sum counted from anthesis.

111×10^{10} Bq/mmol, CEA, France; [^{3}H]adenine, specific activity 77×10^{10} Bq/mmol, CEA, France), used as internal standards, were added to the extract which was stirred overnight at 4°C in darkness. The extract was passed through a Millipore prefilter connected with a Sep Pak C 18 cartridge (Waters, USA). The prefilter and the Sep Pak cartridge were rinsed with 10 mL of 80% methanol. The bulk eluates were reduced to about 50 μL by rotary evaporation, taken up with 1 mL acidified water (0.6 M acetic acid), and injected into a reverse phase HPLC column (Lichrospher C18, Merck, RFA: dimension: 250 × 4.6 mm; particle diameter: 5 μm). Elution was by means of an acidified water:methanol gradient on a Beckman 114 M HPLC system (flow-rate: 0.4 ml/min). Tributylamine (10 mM) was added in solvents to improve separation. Fractions of 400 μL (1 min elution time) were collected in 1.5 mL Treff microcentrifuge test tubes (Treff AG, Switzerland) and evaporated to dryness in a Speed-Vac concentrator (Savant, USA). All fractions were then methylated with diazomethane and taken up in 1.5 mL distilled water. For each fraction two aliquots were taken. One was subjected to liquid scintillation spectrometry for measurement of the recovered radioactivity, the other one to immunoassay. The average recoveries were, respectively, 60% IAA, 84% ABA, and 85% adenine.

ELISA Procedure

Rabbit antihormone antibodies were obtained and characterized as already described (13, 15, 23). Polystyrene microtitration plates (Nunc, Denmark) were coated with hormone conjugate (5 μg/mL in 0.05 M carbonate/bicarbonate buffer, pH 9.6). A limited amount of specific antibody and hormone standard or sample were then added. The plates were incubated for 1 h at 4°C in darkness. During this period, competition occurred for antibody between hormone bound to the plates and free hormone in solution. After four washings with purified water containing 0.1% Triton X-100, antihormone antibody bound on the plates was quantified by the means of the avidin-biotin interaction system: the plates were incubated for 1 h with an excess of biotinylated goat anti-rabbit antibody, washed, and subsequently incubated for 1 h with avidin-alkaline phosphatase conjugate. After four washings to eliminate the excess of conjugate, para-nitrophenyl phosphate (1 mg/mL in 1.0 M diethanolamine buffer [pH 9.8], supplemented with 0.01 M $MgCl_2$) was added, and the phosphatase activity bound to the plate was measured spectrophotometrically at 405 nm with a MR 600 (Dynatech, USA) spectrophotometer. The results were analyzed with a Macintosh 2 (Apple, USA) microcomputer. Calculations were made by reference to a calibration curve established on each microtitration plate by a curvilinear regression of magnitude 4 obtained from the average of four standard curves. Hormone levels measured were deduced from the amount of added hormone as radioactive standard.

Presentation of Data

Plants were grown in field where environmental factors, especially temperature, vary from one day to another. As oilseed rape growth is closely correlated to temperature (14), the presentation of data on a chronological scale would not give enough information. We therefore decided to present all our results on a mean day temperature (>0°C) sum scale, thus giving information on both time and temperature. Moreover, the precise pod age, counted from anthesis onward, will be indicated on the upper abscissa. Correspondence between pod age, chronological scale, and mean day temperature scale are given in Table I.

RESULTS

IAA (Fig. 1) levels rose rapidly during the first 4 d from 300 pmol/g DW to over 6100. From 9 to 19 d old pods, auxin levels were relatively stable at an average of 860 pmol/g DW. During maturation, IAA levels rose to 1450 pmol/g DW by d 26 (295°C mean day temperature sum) and afterward evenly dropped down to 480 pmol/g DW by d 47 (583°C mean day temperature sum).

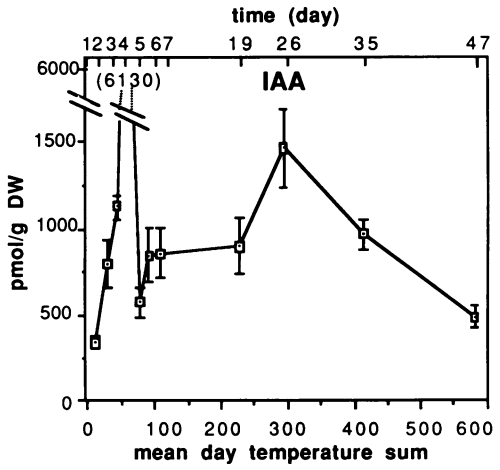

Figure 1. Changes in IAA levels in terminal inflorescence oilseed rape pods during development. Plants were sown in September 1986. Values are expressed in picomoles per gram of dry weight. All are means of five replicates ± SE. Numbers by each point represent pod age (days postanthesis). Bottom abscissa expressed in °C corresponds to the mean day temperature (>0°C) sum, from anthesis. Top abscissa expressed in days corresponds to the pod age.

ABA (Fig. 2) measurements showed large variability during the first week (between 260 and 1000 pmol/g DW). We then observed a peak at d 19 (700 pmol/g DW). Finally, from 295 (d 19) to 583°C mean day temperature sum (d 47) ABA levels rose up to 1600 pmol/g DW. Despite variability there is a tendency to increasing ABA accumulation along pod development.

A bimodal pattern in Z and [9R]Z levels was observed (Fig. 3): during the first week, a high point was observed at age 3 (Z, 840 pmol/g DW; [9R]Z, 420 pmol/g DW) followed by a stable period (around 250 pmol/g DW) at age 5 to 7d. A second peak of accumulation of both compounds occurred at d 19. Moreover, during the period from d 7 to 47, the levels of Z and [9R]Z were remarkably close.

iP and [9R]iP (Fig. 3) were barely detectable during the first week. Their levels began slowly rising at d 7 but then dramatically increased after d 19 up to 850 pmol/g DW at d 26. After reaching this peak, [9R]iP levels dropped just as quickly; levels were at limit of detection in 35 and 47 d old pods. In contrast, iP levels dropped to 600 pmol/g DW at d 35 but went back up to 830 pmol/g DW at d 47.

DISCUSSION

Two periods can be distinguished in the hormone status of pods. Results (first week and then the following maturation period) can be discussed in relation to pod development and correlative morphogenetic and physiological events.

First Week

Pod number and seed number per pod are determined early after flowering (19), and though nutritional factors do play an important role (1, 24), there is evidence that hormones may also operate on these yield components. During this period, important variations of hormonal levels were observed. Both

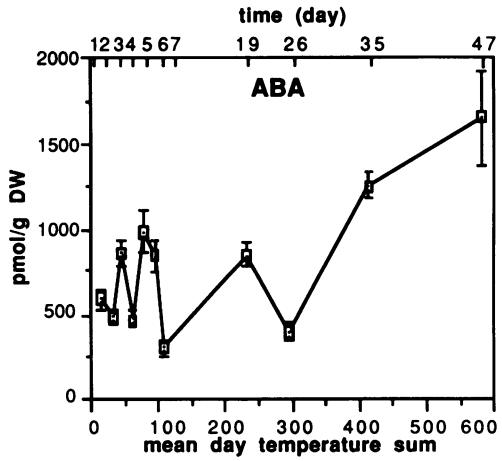

Figure 2. Changes in ABA levels in terminal inflorescence oilseed rape pods during development. Plants were sown in September 1986. Values are expressed in picomoles per gram of dry weight. All are means of five replicates ± SE. Numbers by each point represent pod age (days postanthesis). Bottom abscissa expressed in °C corresponds to the mean day temperature (>0°C) sum, from anthesis. Top abscissa expressed in days corresponds to the pod age.

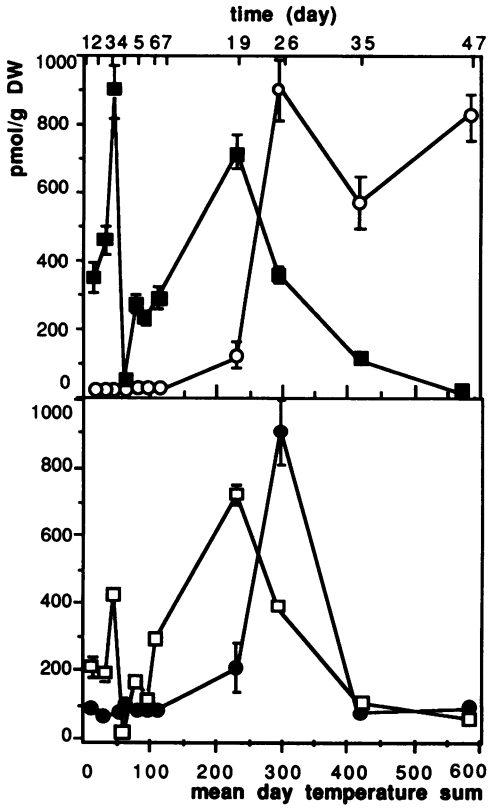

Figure 3. Changes in Z (■), iP (○), [9R]Z (□), and [9R]iP (●) levels in terminal inflorescence oilseed rape pods during development. Plants were sown in September 1986. Values are expressed in picomoles per gram of dry weight. All are means of five replicates ± SE. Numbers by each point represent pod age (days postanthesis). Bottom abscissa expressed in °C corresponds to the mean day temperature (>0°C) sum, from anthesis. Top abscissa expressed in days corresponds to the pod age.

Z and [9R]Z peaked on the third day (Fig. 3). These cytokinin levels are correlated with a high rate of cell division in young embryos a short time after fertilization. It is well known that cytokinins are involved in the regulation of cell division. Such phenomenon has previously been described in soybean (7). Lee *et al.* (12) observed that in a cytokinin deficient *Phaseolus* hybrid, embryonic cell division stopped at 4 cell stage. They succeeded in reestablishing cell division with exogenous cytokinin. Moreover, high cytokinin levels partially determine the final pod number by reducing flower and pod abortion. Carlson *et al.* (5) assumed that soybean flower abortion in some circumstances is related to a deficiency of root-produced cytokinins. These results suggest that in oilseed rape, early embryogenesis, early young pod development, and pod number may also be under partial cytokinin control.

The level of IAA sharply increased on d 4, but fell just as quickly by d 5 (Fig. 1). IAA is thought to be involved in the regulation of assimilate partitioning, and this has been described more precisely in soybean seed filling by Brenner (3). Moreover, Brun *et al.* (4) showed that sink strength of soybean flowers was determined during the first days after flowering. Sink intensity decreased shortly after anthesis, and rose back up a few days later. Only aborting flowers did not recover

their sink strength. It is possible that hormones, particularly IAA, are involved in determining sink strength. Similarly in oilseed rape, IAA could prevent flower or pod abortion, and therefore improve pod number. Furthermore, Inanaga and Kumura (11) published evidence on oilseed rape showing that seed number per pod was essentially dependent on IAA and not assimilate supply. Thus, IAA could be related, not only to pod number, but also to seed number per pod.

Maturation Period (7 d Onward)

During this period two phases that are separated at the 300°C mean day temperature sum can be identified. During the first phase, the pods are heterotrophic: pod development depends on assimilates supplied from other parts of the plant. However, pods become autotrophic during the second phase, photosynthesis occurring in pod wall (14). Attention must be focused on the fact that hormonal analyses generally show the same high point.

IAA level reached a second peak in 26 d old pods (300°C mean day temperature sum) (Fig. 1). Positive influence between IAA and seed filling has been previously reported (3). Moreover, it is probable that IAA synthesized in the seeds enhances assimilate accumulation in fruit. In oilseed rape, IAA produced by seeds could be partially responsible for pod wall development. As pod development proceeds (300°C mean day temperature sum onward), its influence would decrease as pods become self-dependent for carbohydrate supply. Meanwhile it is remarkable that even at d 49, IAA levels are always higher than 500 pmol/g DW. Therefore, IAA could also have an effect on seed filling.

ABA accumulated during the autotrophic phase of the maturation period. Although hormonal levels were measured in whole pods, they will be mainly discussed in relation to seed maturation. Indeed, many studies have provided evidence for ABA involvement in seed maturation. ABA could operate on preferential orientation of assimilate flux to seeds (2). Furthermore, it may partially influence protein accumulation in soybean embryos (8). Quatrano (20) demonstrated a correlation between ABA and cruciferine mRNA in oilseed rape. Moreover, ABA accumulation has been related to seed dehydration and germination inhibition; meanwhile, the precise role of ABA on germination has not yet been definitely established (9). Our results showed an increasing ABA accumulation especially during the second part of pod maturation period during which seed filling occurs. Therefore, and despite the fact we did not measure separately the different parts of seeds or pods, we suggest that ABA probably plays an important role in seed development and more precisely in seed filling of oilseed rape. Thus, ABA could be a component of seed specific weight.

From 7 to 47 d, the kinetics of Z and [9R]Z levels were similar. They both reached a high point in 19 d old pods. The highest iP and [9R]iP concentrations were measured in the next sample at d 26 (295°C mean day temperature sum), while Z and [9R]Z decreased rapidly. In the last sample (d 47) only [9R]IP was detected. Total cytokinin reached progressively climax at d 26, then progressively decreased (Fig. 3). These changes suggest that modification of cytokinin metabolism occurs in pods between 250 and 300°C mean day temperature sum. [9R]iP is known to be a precursor of [9R]Z and then Z (10, 18). Thus, the accumulation of iP forms cannot be explained by degradation of Z and [9R]Z. It is likely that the iP or [9R]iP accumulate when the conversion to their respective Z forms slows without a concomitant reduction in precursor biosynthesis.

For the first time as many as six endogenous hormonal levels have been measured in pods of field-grown plants, with precise HPLC and ELISA methods. They show that endogenous hormonal levels may change in a short time (1 d) within a very wide scale, especially during the first week development. They allow the hypothesis of close relationship between hormone levels and pod development, and probable correlations between PGR and main yield components.

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