Cytokinin Activity in *Lupinus albus* L.

IV. DISTRIBUTION IN SEEDS

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ABSTRACT

Endogenous levels of cytokinin activity were examined in *Lupinus albus* L. seed at intervals of 2 weeks after anthesis using the soybean callus bioassay. High levels of cytokinin activity per gram seed material were present in the seeds at 2, 4, and 6 weeks after anthesis. The cytokinin activity per gram seed material was low at 8 and 10 weeks after anthesis. Cytokinin activity associated with each seed was greatest at 6 weeks after anthesis. The majority of the activity in the seeds at 4, 6, and 8 weeks after anthesis was in the endosperm. Cytokinin activity was also detected in the testas and embryos at 4, 6, 8, and 10 weeks, and the suspensors at 4 weeks. Column chromatography of extracts of the different seed fractions on Sephadex LH-20 indicated that the cytokinins present coeluted with zeatin, zeatin riboside, and the glucoside cytokinins. It is suggested that cytokinins are accumulated in the seeds and are stored in the endosperm mainly in the form of ribosides and glucosides of zeatin. The reduction in cytokinin activity in the seed coincides with the reduction in endosperm volume and embryo growth and suggests that these compounds are utilized during the course of seed maturation.

The fruits of the white lupin (*Lupinus albus* L.) have recently been investigated intensively with respect to the biochemical (2, 12, 21), ultrastructural (9), and hormonal (6, 8) aspects of fruit development. Both quantitative and qualitative changes have been shown to occur in the cytokinin levels in white lupin seeds (8).

Cytokinins are known to occur in the testas, embryos, and endosperms of a number of seeds (3, 4, 16, 25). High levels of gibberellins and auxins are present in the endosperm of pea seeds (10), and it has been reported (5) that high cytokinin levels in these seeds are present when there is a high proportion of liquid endosperm. The quantitative and qualitative changes in cytokinins in different parts of a legume seed have not been investigated.

MATERIALS AND METHODS

Plant Material. *L. albus* L. plants were grown as previously described (7). The flowers on the primary inflorescences were labeled at anthesis. Fruits were harvested at 2, 4, 6, 8, and 10 weeks after anthesis. Seeds were excised and their fresh weights recorded. At 2 weeks intact seeds were analyzed. At subsequent intervals seeds were separated into different fractions depending on their age. Four-week-old seeds were divided into testa and endosperm fractions. A small incision was made in each seed and the endosperm blotted on thin strips of Whatman No. 1 filter paper. The weight lost by the seed after the removal of the endosperm was regarded as being representative of the weight of the endosperm. At this time the embryo was small and attached to the embryo sac wall by a suspensor. In a separate experiment, the suspensors and embryos were dissected from the seeds and the embryo detached from the suspensor. The embryos and suspensors were rinsed in distilled H₂O to remove soluble endospermic fluid and then extracted in 5 ml 80% ethanol. Six-week-old seeds were divided into testa, endosperm, and embryo fractions. The liquid endosperm was collected as described above. Cellular endosperm was carefully removed from around the embryo with a wick of filter paper. The testa and embryos were weighed and an estimate of the endosperm weight obtained. Eight-week-old embryos were separated into testa, embryo, and endosperm fractions. The small amount of cellular endosperm around the embryo was removed as described above. At 10 weeks endosperm was absent and only embryo and testa samples were collected. After collection all plant material was stored at −20 C until analyzed.

**Extraction and Purification of Cytokinins.** The cytokinins present in testas and embryos were extracted and purified as previously described (7). Dowex 50W-X8 cation exchange resin was used at a resin to plant material ratio of 2:1. Intact seeds at 2 weeks after anthesis, embryos and suspensors at 4 weeks, and embryos at 6 weeks were ground in 80% ethanol in a glass homogenizer. The extracts were filtered, concentrated to dryness under vacuum, and the residue taken up in 2 ml 8% ethanol and applied directly to paper chromatograms. For column chromatography, these extracts were concentrated and the residue dissolved in 1 ml of 35% ethanol and applied directly to a Sephadex LH-20 column. The strips of filter paper on which endosperm had been collected were washed with 500 ml 80% ethanol. This extract was then filtered, concentrated to dryness, and the residue taken up in 2 ml 80% ethanol and applied directly to paper chromatograms.

**Chromatographic Separation.** When separated on paper, the extracts were stripped and loaded onto Whatman No. 1 chromatography paper. The chromatograms were developed with isopropyl alcohol-25% ammonium hydroxide-water (10:1:1, v/v), air-dried, and then divided into 10 R₂ zones. These zones were assayed for cytokinin activity using the soybean callus bio assay (17). Samples fractionated by means of column chromatography were separated on Sephadex LH-20 (90 × 2.6 cm) and eluted with either 35% ethanol or 20% ethanol at a flow rate of 15 ml/h (1). Forty-ml fractions were collected, transferred to Erlenmeyer flasks, air-dried, and then assayed for cytokinin activity.

RESULTS

The white lupin seed increased in fresh weight between 2 and 8 weeks after anthesis (Fig. 1B). During this time the changes in fresh weight of the embryo, endosperm, and testa (Fig. 1C) were accompanied by morphological changes in the seed (Fig. 1A). In 4-week-old seeds the embryo was attached by means of a multicellular suspensor to the testa. The embryo was surrounded by a semiliquid, semicellular endosperm which, at this time, contributed approximately 21% of the fresh weight of the seed. The seed increased in fresh weight between 4 and 8 weeks after anthesis.
During this period, the developing embryo displaced the endosperm. At 8 weeks the embryo represented approximately 44.9% of the fresh weight of the seed. The endosperm was absent at 10 weeks, and the embryo contributed about 52.2% of the fresh weight of the seed.

In Figure 1, D, E, and F, the averaged results of bioassays of extracts purified by both paper and column chromatography are expressed as µg zeatin equivalents. These results show that the total amount of cytokinin activity changed during growth and development. At 6 weeks the highest level of cytokinin activity was recorded irrespective of whether the results were expressed on the basis of activity per gram seed material analyzed or on the basis of activity per seed. The results are discussed in terms of cytokinin activity (µg zeatin equivalents) per seed or portion of the seed.

Analysis of 2-week-old seeds for cytokinins showed that low levels of cytokinin activity were present (Fig. 1E). By 4 weeks after anthesis, the total level of activity was increasing (Fig. 1E). A higher level of activity was apparently associated with the endosperm than with the testa (including both embryo and suspensor) (Fig. 1F). Separate analysis of paper chromatograms of embryo and suspensor extracts showed that a large proportion of the activity at this time was associated with these organs (Fig. 2). A higher level of activity was present in the suspensor than in the embryo.

The highest level of cytokinin activity was detected in 6-week-old seeds (Fig. 1, D and E). At this time compounds exhibiting cytokinin activity could be detected in the embryo, endosperm, and testa (Fig. 1F). Most of the activity was still located in the endosperm (Fig. 1F).

By 8 weeks after anthesis, the cytokinin activity in the seeds had started decreasing (Fig. 1E). This decrease appeared to be due to a decrease in the level of activity in endosperm extracts. The level of cytokinin activity continued to decrease and was low in 10-week-old seeds. At 10 weeks most of the activity in the seed was in the testa (Fig. 1F).

Following column chromatography on Sephadex LH-20, five different peaks of cytokinin activity were detected in extracts of 2-week-old seed (Fig. 3). Two peaks coeluted with authentic zeatin and zeatin riboside while the remaining peaks had elution volumes of 200 to 280 ml, 360 to 480 ml, and 800 to 840 ml, respectively (Fig. 3).

In the testas, four peaks of cytokinin activity were detected at 4, 6, 8, and 10 weeks after anthesis. At 6 weeks after anthesis, two of these coeluted with zeatin and zeatin riboside. The remaining peaks eluted off Sephadex at 320 to 360 ml and 360 to 440 ml (Fig. 4).

Cytokinin activity in the embryos coeluted primarily with zeatin riboside at 6, 8, and 10 weeks. At 6 weeks activity was also detected at elution volumes of 200 to 240 ml, 280 to 320 ml, and 800 to 840 ml (Fig. 5).

High levels of cytokinin activity were present in both fast and slow moving fractions of paper chromatograms of endosperm extracts (Fig. 6). The chromatograms were therefore divided into
zeatin riboside and zeatin glucoside on Sephadex LH-20 eluted with 35% ethanol. In 4- and 8-week-old seeds low levels of activity coeluting with zeatin were also recorded, while in 4-week-old seeds some activity was detected in fraction A at the elution volume 360 to 440 ml (Fig. 7).

**DISCUSSION**

The present results show that white lupin seed development is accompanied by an increasing level of cytokinin activity up to 6 weeks after anthesis, thereafter it declines. This is in agreement with previous results obtained for the white lupin (8) and for *Curcubita pepo* seeds (11). Gupta and Maheshwari (11) reported that cytokinin levels were low in both very young and very old *C. pepo* seeds. However, it has been shown that in *Citrullus lanatus*, the cytokinin level in the whole seed is maximal at 11 days after pollination whereas the seed size increase is most rapid between 4 and 9 days after pollination (22). This observation led to the suggestion that rather than contributing to the growth of the seed, the increased cytokinin levels are a mere accompaniment of growth (22). In white lupin seeds, increasing levels of activity...

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**FIG. 2.** Cytokinin activity in 4-week-old embryos and suspensors of white lupin seeds. Embryos and suspensors removed from 30 seeds were ground in a glass homogenizer in 80% ethanol. Ethanolic extract was reduced to dryness under vacuum and taken up in 1 ml 80% ethanol and streaked onto Whatman No. 1 chromatography paper. Chromatograms were run in isopropyl alcohol-25% ammonium hydroxide-water (10:1:1 v/v), divided into 10 R, strips and assayed. Z: zeatin; ZR: zeatin riboside. Broken lines represent confidence limit at P = 0.01.

**FIG. 3.** Cytokinin activity in 0.88 g fresh weight of seed harvested at 2 weeks after anthesis. Seed material was fractionated on a column packed with Sephadex LH-20 and eluted with 35% ethanol. Z: zeatin; ZR: zeatin riboside; ZG: zeatin glucoside. Broken line represents confidence limit at P = 0.01.

Two fractions, fraction A (Rf 0.0-0.5) and fraction B (Rf 0.5-1.0). Three major peaks of activity were detected after elution of activity present in fraction A through a Sephadex LH-20 column eluted with 20% ethanol. One peak coeluted with zeatin riboside, while the remaining peaks had elution volumes of 240 to 320 ml and 440 to 520 ml, respectively. Cytokinins in fraction B coeluted with...

**FIG. 4.** Cytokinin activity in extracts of 2.5 g testa material following fractionation on a column packed with Sephadex LH-20 and eluted with 35% ethanol. Testas were collected from 6-week-old seeds. Z: zeatin; ZR: zeatin riboside; ZG: zeatin glucoside. Broken line represents confidence limit at P = 0.01.

**FIG. 5.** Cytokinin activity in an extract of embryos harvested at 6 weeks after anthesis. Embryo extract was fractionated on a Sephadex LH-20 column eluted with 35% ethanol. At 6 weeks 0.8 g embryo material was extracted. Z: zeatin; ZR: zeatin riboside. Broken line indicates confidence limit at P = 0.01.
FIG. 6. Cytokinin activity in 0.25 g endosperm material collected from seeds harvested at 4, 6, and 8 weeks after anthesis. Extracts were chromatographed on Whatman No. 1 chromatography paper, run with isopropyl alcohol-25% ammonium hydroxide-water (10:1:1 v/v). Z: zeatin; ZR: zeatin riboside. Broken line represents confidence limit at $P = 0.01$.

FIG. 7. Cytokinin activity in 0.25 g endosperm material following fractionation on Sephadex LH-20 eluted with 20% (A) and 35% (B) ethanol. Extracts of endosperm collected from 6-week-old seeds were initially chromatographed on paper. Paper chromatograms divided into two fractions: fraction A, $R_f$ 0.05 to 0.5; and fraction B, $R_f$ 0.5 to 1.0, and the activity eluted and run through Sephadex LH-20. Z: zeatin; ZR: zeatin riboside; ZG: zeatin glucoside. Broken line represents confidence limit at $P = 0.01$. 

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accompany growth only up to 6 weeks after anthesis. There is a
decline in cytokinin activity when the seeds increase rapidly in
fresh weight. Ultrastructural studies of the embryonic cotyledons
during this phase of growth show that the lupin embryo begins to
synthesize storage proteins actively during this phase of growth
(9). It is possible that cytokinins can contribute in some way to
this process in white lupin embryos at this time. It has been
suggested that seeds may synthesize the hormones responsible for
pod wall growth and development (10) and previous studies have
shown that the cytokinin level in the white lupin pod wall increase
with fruit age (8). Loss in cytokinin activity by the seed may
therefore also be viewed as a transference of these compounds to
the pod wall. Although some cytokinins in the pod wall may
originate in the seeds, recent studies of cytokinin levels in pea
fruits cultured in vitro indicate that these compounds are not
synthesized within the fruit system during seed growth (15, 24).
Cytokinins are apparently imported from other parts of the plant,
indeed cytokinin activity has been detected in the sap passing into
white lupin fruits (8).

Between 2 and 6 weeks after anthesis, the embryo is a very
small meristematic structure (9). It increases markedly in size and
fresh weight after this time and the cotyledon cells become the
major storage organ for protein, carbohydrate and lipid (9). In the
“competing sinks” hypothesis (18) it has been suggested that these
nutrients, needed for the building of new tissues, limit fruit growth,
and that the high concentrations of hormones found in the seed
are necessary in order to create a strong physiological sink capable
of competing with the remainder of the plant for nutrients (18).
Cytokinins have been implicated in such an hypothesis (18), and
it has been demonstrated that sugars and amino acids can be
transported to regions of high cytokinin levels (20). The present
results indicate that there is a build up of cytokinins in the seed
tissues. This is particularly noticeable in the endosperm between
4 and 6 weeks after anthesis, a period during which it has been
established that pools of sugars and amino acids are formed in
white lupin seeds (2). The major utilization of carbon and nitrogen
occurs between 4 and 8 weeks after anthesis during which time
there is rapid seed growth (2). This is followed by a period when
polysaccharide, protein, oil, and wall reserves are laid down in the
developing embryo (2, 9).

Liquid endosperm accumulates in the seed at an early stage of
development and, although it does not become fully cellular, its
liquid content disappears as the embryo encroaches on the endo-
permic cavity. High levels of cytokinin activity are associated with
the endosperm rather than with the developing embryos. As
cytokinins stimulate both cell division and cell expansion (19),
tissues enclosed in a fluid containing such growth promotors
must regulate the availability of these compounds. It has been
suggested that the suspensor can regulate the supply of growth
substances passing to the embryo during the early stages of
development (23). Cytokinins were present in white lupin suspensi-
sors (Fig. 2). This observation and the “transfer cell” type structure
of the suspensor cell walls (unpublished data) suggest that these
cells may have a regulatory function. At later developmental
stages the embryo is completely encased in cellular endosperm,
and the cytokinins are thus not in direct contact with the embryo.

The cytokinins present in the seed tissues coelute with zeatin
and zeatin riboside following column chromatography. Cell divi-
sion inducing compounds are also present at elution volumes 200
to 320 ml, 320 to 440 ml, and 800 to 840 ml, when fractions are
eluited with 35% ethanol and at an elution volume of 440 to 520
ml following elution with 20% ethanol. The activity at elution
volumes 320 to 440 ml and 440 to 520 ml after column chroma-
tography in 35% and 20% ethanol, respectively, coelutes with
zeatin glucoside. The glucosylation of cytokinins has been sug-
gested to be a means of inactivation (13, 26) and thus storage (8,
14) of these compounds. The cytokinins coeluting with the glu-
cosides of zeatin present in white lupin seed tissues may represent
inactivated and/or stored cytokinins.

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