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Growth-Promoting Factors in Young *Lupinus* Seeds

By

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Since it was reported that coconut milk, a liquid endosperm of *Cocos nucifera*, had growth-promoting activities for isolated young embryos[1] and cultured tissues[2)3)], the purification of such active principles has been made[4)5)6)], and several active substances were isolated as growth-promoting factors for cultured tissues[7)8)9)]. However, it has not been demonstrated yet whether or not these substances have embryo factor activity, i.e., growth-promoting activity for young embryo. Moreover the study on embryo factor has remained without any advances.

In the course of studies of growth factor present in young *Lupinus* seeds it was found that ethanol extract of seeds had not only embryo factor activity but also cytokinin one[10]. The present study is concerned with these active factors, and their physiological roles in plant growth are discussed.

Materials and Methods

i) *Preparation of crude extract*: Young seeds of *Lupinus luteus* harvested between 2 and 3 weeks after pollination were immersed in equal volume of 80 per cent ethanol or methanol. After a few days extraction the mixture was filtered and the residue was extracted again. Combined ethanol solution was evaporated with a rotary evaporator at 40°C. The residue was referred as the crude extract. For convenience the amounts of the extract and of the purified fractions were expressed also in terms of gram equivalents (GE). One GE designates the amount of the extract derived from 1 g of *Lupinus* seeds. The crude extract is generally dissolved with water at a concentration of 10 GE/l and stocked in a freezer.

ii) *Assay of embryo factor*: The crude extract or its partially purified fractions to be tested for their embryo factor activity were added to the basal medium composed of inorganic salts[11], 8 g agar and 20 g sucrose per liter. The pH of the media was adjusted with N HCl and N Na_2CO_3 and measured by a Beckmann Electrode pH Meter. The media were distributed in aliquots of 10 ml in test tubes and autoclaved at 1.0 kg/cm^2 overpressure for 10 minutes, unless otherwise stated.

The techniques of the isolation of young embryos of *Datura tatula* were the same as reported by van Overbeek et al[1]. Young heart-shaped embryos, 0.2–0.3 mm in length, were implanted 1 mm below the agar surface in test tubes. Ten test tubes each with one embryo were used for each test lot. Test tubes were maintained in darkness for five days at 30°C. Embryo factor activity was expressed by the final length of embryos.

iii) *Assays of cytokinin*: Cytokinin activity in test materials was appreciated by three kinds of assay systems, i.e., 1) the growth of calluses of tobacco and carrot, 2) the growth of radish leaf disks, and 3) a chlorophyll preservation test using radish cotyledons.

Tobacco calluses (*Nicotiana tabacum* var. Wisconsin No. 38) originally derived from pith were cultured on a medium for stock culture at 27°C under continuous fluorescent light. At frequent intervals of 4 weeks, about 50 mg portions of them were subcultured. Callus of carrot (*Daucus carota*) was obtained from the second phloem of root. The procedures for stock culture were the same as those of tobacco tissue. The basal medium for culture was composed of modified White's

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salts[11], 8 g agar, 20 g sucrose, 3 mg glycine, 0.5 mg nicotinic acid, 0.1 mg thiamine•HCl, 0.1 mg pyridoxine•HCl, 500 mg NH_4NO_3, 100 mg *myo*-inositol, 100 mg casein hydrolysate and 1 mg indoleacetic acid per liter, but in the stock culture 0.1 mg/l kinetin was supplemented additionally. Materials to be tested were added to the basal medium and then the test media were distributed in aliquots of 12 ml in every tube. The tubes were autoclaved at 1.0 kg/cm^2 for 10 minutes. Small callus pieces (15–25 mg) obtained from the stock cultures of tobacco and carrot were implanted on the agar surface. They were maintained under the same conditions as the stock cultures for 4 weeks. The cytokinin activity was expressed by the increase of final fresh and dry weights of tissues.

Cytokinin activity was estimated by the growth of radish leaf. This assay system was essentially the same as established by Kuraishi[12]. Ten sheets of disks (5 mm in diameter) were floated in the nutrient media[12] with and without test substance in a small Petri dish. They were maintained for 24 hours under continuous illumination of fluorescent light at 28°C. Cytokinin activity was expressed by fresh weight and diameter of leaf disks.

Cytokinin activity was also detected by the inhibition of chlorophyll degradation. This assay system was essentially the same as used by Loeffler and van Overbeek[13]. For each test lot, ten green cotyledons harvested from field-grown radish seedlings were placed on a moist filter paper in a Petri dish. Two disks of filter paper (5 mm in diameter) dipping with the test solution were placed on flat area of each cotyledon. Petri dishes were maintained in darkness at 27°C for three days. If the solution contains cytokinins, the area treated with the test solution remained green but the rest area turned yellow. Thus cytokinin activity was determined by visual estimation and expressed by arbitrary units from 1 to 5.

Results and Discussion

Embryo factor

Experiments with the crude extract : The crude extract obtained from young *Lupinus* seeds stimulated the growth of young embryos. Prior to the following studies, determination of suitable level of the extract was required. Embryos were cultured on media with varying concentrations of the extract (0.01–1 GE/ml), and their growths were observed. With increase of the extract till 0.1 GE/ml, a progressive promotion to embryo growth was observed. At a level of 0.1 GE/ml embryos grew most vigorously (Fig. 1). Higher concentrations were rather inhibitory.

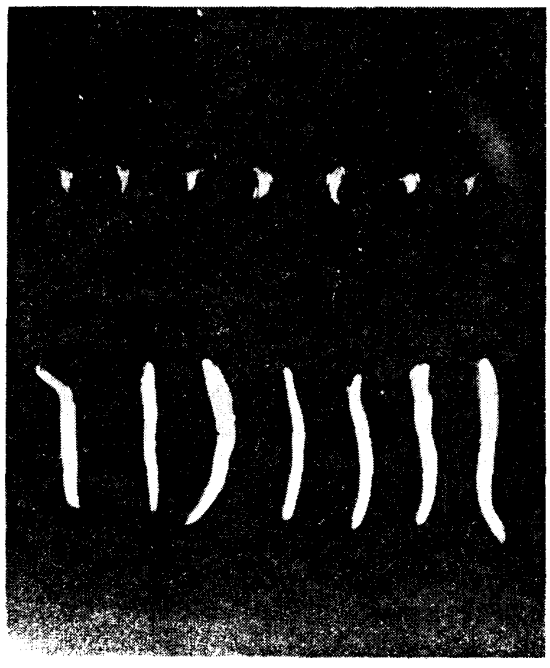


Fig. 1. Effect of the crude *Lupinus* extract on the growth of young embryos of *Datura*. Upper, control; lower, *Lupinus* extract.

Solomon reported[14] that autoclaved coconut milk was inactive or inhibitory compared with filter-sterilized one, and this inhibition was caused by a inhibitor produced during autoclaving. In the case of *Lupinus* extract, autoclaving affected the effectiveness slightly. The growth of embryos on medium with autoclaved extract was inferior, though insignificantly. Such slight inhibition seemed to be reduced by ether extraction of inhibitor, because it was extracted with ether but active factors were not.

Partial purification: Purification of embryo factor was carried out by Neuberg precipitation. The procedures were the same as employed by Mauney et al[5]. The crude extract (200 GE, 5.6 g of dry residue) was diluted with water to 200 ml. The pH was adjusted

to 8.2 with a saturated sodium carbonate solution. Mercuric acetate (25 per cent aqueous solution) was added dropwise to the solution. A cream colored precipitate occurred. Adjustment of the pH with sodium carbonate and addition of mercuric acetate were repeated again. Such alternate additions were continued further several times until an orange colored precipitate appeared. Then 50 ml of ethanol was added to this mixture, and kept in refrigerator for 24 hours. After centrifugation, the supernatant was decanted (Fraction 1), and the residue was suspended with 80 per cent ethanol and recentrifuged. The precipitate was suspended with water. The suspension was treated with H_2S, and the mercuric sulfide was removed by centrifugation. The supernatant was concentrated and yielded 1.35 g of syrup (Fraction 2). Fractions 1 and 2 were tested for their embryo factor activity at a level of 0.1 GE/ml.

A strong activity was found in Fraction 2, though considerably less effective than the crude extract. In the Fraction 1 only a weak activity was found. This results showed that most of active principle was precipitated by Neuberg precipitation.

Fraction 2 was further purified with ion-exchange resin. Syrup (1.4 g, 200 GE) was dissolved in 100 ml of water. The solution was passed through a column of Amberlite IRA 400 (carbonate form, and 30 ml). After the column was washed with 200 ml of water, the effluent and washings were combined and evaporated to dryness, resulting in 0.4 g of dry substance (Fraction 3). On the other hand adsorbed substances were eluted from the column with 400 ml of 2 N acetic acid. Upon evaporation, 0.64 g of dry substance was obtained (Fraction 4).

Fractions 3 and 4 were tested at a level of 1.4 GE/ml, namely 320 and 450 mg/l respectively. Lengths of embryos were 1.04, 3.17, and 5.19 in control and Fractions 3 and 4 respectively. The eluate was more effective than the effluent. The former was employed for additional purification.

Fraction 4 (318 mg dry residue, 100 GE) was dissolved in 100 ml of water. The solution was poured into a column of Dowex 50 W×8 (30 ml, 50–100 mesh, and H^+ form). The column was washed with 200 ml of water. Evaporation of combined filtrates yielded 71 mg of dry residue (Fraction 5). The adsorbed substances were eluted with 6 N ammonia. From the eluate 234 mg of dry residue was obtained (Fraction 6).

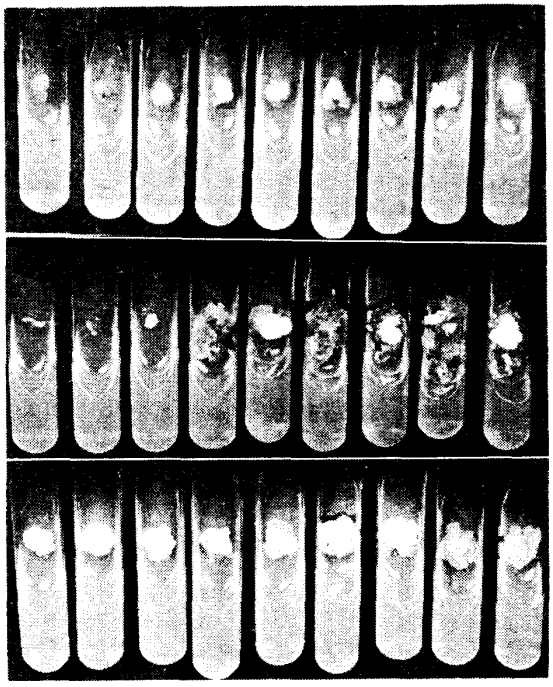


Fig. 2. Effects of the *Lupinus* extract and kinetin on the growth of tobacco calluses. Upper, control ; middle, *Lupinus* extract in 10 GE per liter ; lower, kinetin in 0.03 mg per liter.

Fractions 5 and 6 were tested at a level of 0.14 GE/ml, i.e., 32.8 and 10.4 mg/l respectively. The lengths of embryos were 1.25, 3.86 and 4.57 in control, Fractions 5 and 6 respectively. The eluate was more active than the effluent. This result shows that the larger active principle was adsorbed on Amberlite IRA 400 (CO_3^{--} form) and Dowex 50 W×8 (H^+ form), and that embryo factor activity in the crude extract seemed to consist of several active components.

Cytokinin

Since Miller et al. found a cell-division factor, kinetin, from DNA hydrolysate[15], the isolation of kinetin-like factor from plant materials had been attempted[13)16)17]. Recently Letham succeeded to get a cytokinin from *Zea mais*[18]. In *Lupinus* seeds, cytokinin activity was shown and it was studied from another view point apart from embryo factor.

Crude extract : As shown in a previous paper[10], the crude *Lupinus* extract used at a level of 10 GE per liter was most efficient in the growth by cell-division of tobacco callus (Fig. 2). This indicates that this level is ten times lower than that used in embryo culture. A preliminary experiment for determination of favorable

Table 1 Effect of various concentrations of the crude *Lupinus* extract on the growth of carrot calluses.

Additives	Conc. (mg/l)	Fresh weight per piece (mg)	Dry weight per piece (mg)
-	-	141.7	17.7
Lupinus extract	125*	211.3	26.0
//	250	256.2	28.7
//	500	294.3	32.3
//	1250	181.5	22.2
//	2500	114.5	14.8
Kinetin	0.1	318.0	36.0

* 125 mg of the extract was obtained from 5 g of fresh *Lupinus* seeds (5 GE).

Table 2. Visual estimation of green color remaining in radish cotyledon. The color of the area in application of test substance was rated 1-5 in comparison to the color of cotyledons treated with kinetin (10 mg/1).

Test material	Estimation
Water	0
Crude extract (2 GE/ml)	4.2
// (20 GE/ml)	5.0
Eluate from Dowex 50 (10^4 mg/l)	4.1
Silver-precipitated fraction (10^4 mg/l)	5.0
Kinetin (1 mg/l)	4.6
// (10 mg/l)	5.0

level of the extract was attempted for carrot callus. Calluses were cultured on the media containing various concentrations of the crude extract. As shown in Table 1, calluses on the basal medium without the extract was not able to continue their growth. The minimum concentration showing cytokinin activity was 5 GE/liter (125 mg dry matter/l). The largest growth was obtained at the concentration of 500 mg/l (20 GE per liter). This concentration is five times lower than that needed in embryo culture. Higher concentration of the extract was inhibitory. Kinetin used as another control was also very effective to the continuous growth of tissue.

Tobacco and carrot tissues cultured on the basal medium were friable and composed of loosely packed larger cells, while tissues supplied with kinetin or the extract were generally composed of numerous meristematic smaller cells. This indicates that the increases of fresh and dry weights of tissues by the extract are apparently caused by increasing rate of cell-division.

Crude *Lupinus* extract was slightly stimulative to the growth of radish leaf disks at a level of 200 GE/l (Fig. 3) and inhibitory to the degradation of chlorophyll of radish cotyledons at 2-20 GE/ml (Table 2).

Partial purification : The crude extract (27 g of dry residue) obtained from 2 kg of *Lupinus* seeds was treated with Neuberg precipitation as described previously. Cytokinins were precipitated, and supernatant was inactive.

The precipitated fraction (2.8 g of dry residue) was further purified with ion-exchange resin. The solution was poured into a column of Dowex 50 W×8 (H^+ form), followed by elution with 3 N NH_4OH. The effluent and the eluate yielded 1.1 and 1.2 g of dry substance respectively. They were tested at the concentration of 100 mg/l.

The eluate was very effective to the growths of tobacco and carrot calluses. It was also effective in leaf growth test at $2 \cdot 10^3$ mg/l (Fig. 3) and in chlorophyll preservation test at 10^4 mg/l (Table 2). These results indicated that *Lupinus* cytokinin, like kinetin, was adsorbed on Dowex 50 and eluted with ammonia water.

The eluate fraction (1.0 g of dry residue) was acidified to pH 3.5 with 3 N H_2SO_4. To the solution was added excessive amount of 10 per cent of silver nitrate solution. The precipitate and the supernatant were separated by centrifugation. The supernatant was discarded because of no cytokinin activity. Then the precipitate was extracted with 0.2 N HCl. The supernatant was decanted and the residual precipitate was discarded. Evaporation of the former produced 0.4 g of dry substance, which was named the silver-precipitated fraction.

This fraction was tested for its cytokinin activity at the concentrations of 10 and 100 mg per liter. A dramatic activity of cell division in tobacco callus was observed (Fig. 4). This fraction at 660 mg/l was also very effective to the growth of radish leaf disks, and this activity was nearly equivalent to that of 1 mg/l kinetin (Fig. 3). In chlorophyll preservation of radish cotyledon this

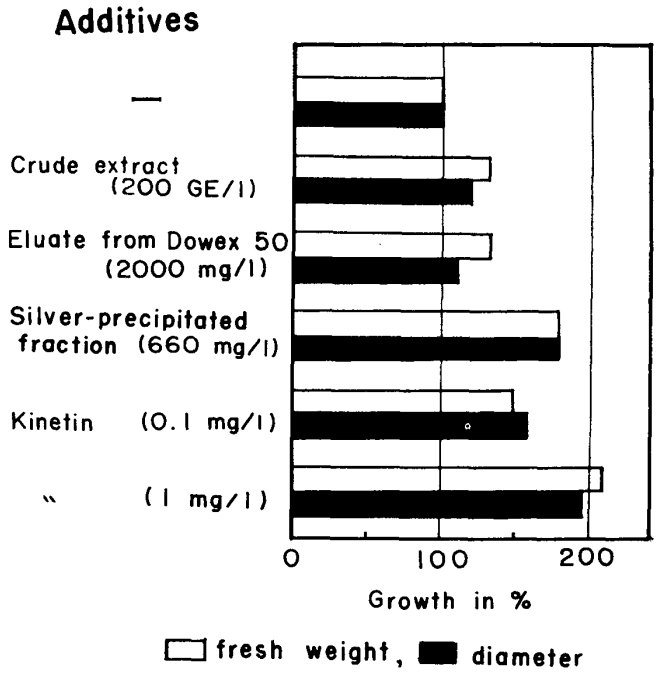


Fig. 3. Effects of the crude extract, purified fractions and kinetin on the growth of radish leaf disks. Growths are shown as per cent of controls.

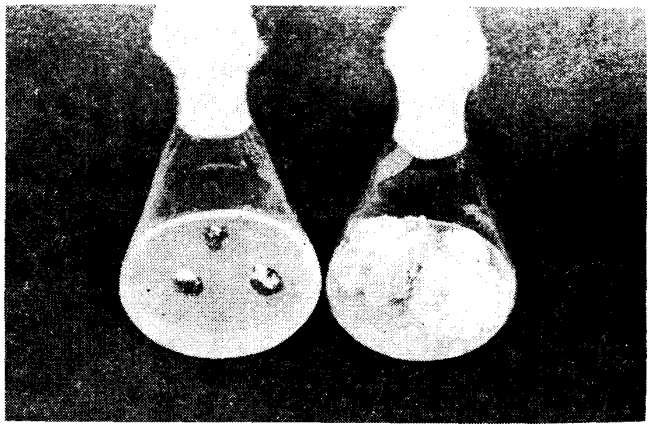


Fig. 4. Tobacco calluses cultured on the media with and without the silver-precipitated fraction (100 mg/l) for 45 days. Left, control; right, silver-precipitated fraction.

fraction showed a strong activity (Table 2). These results indicates that the silver-precipitated fraction has the same physiological activities in plant growth as kinetin does.

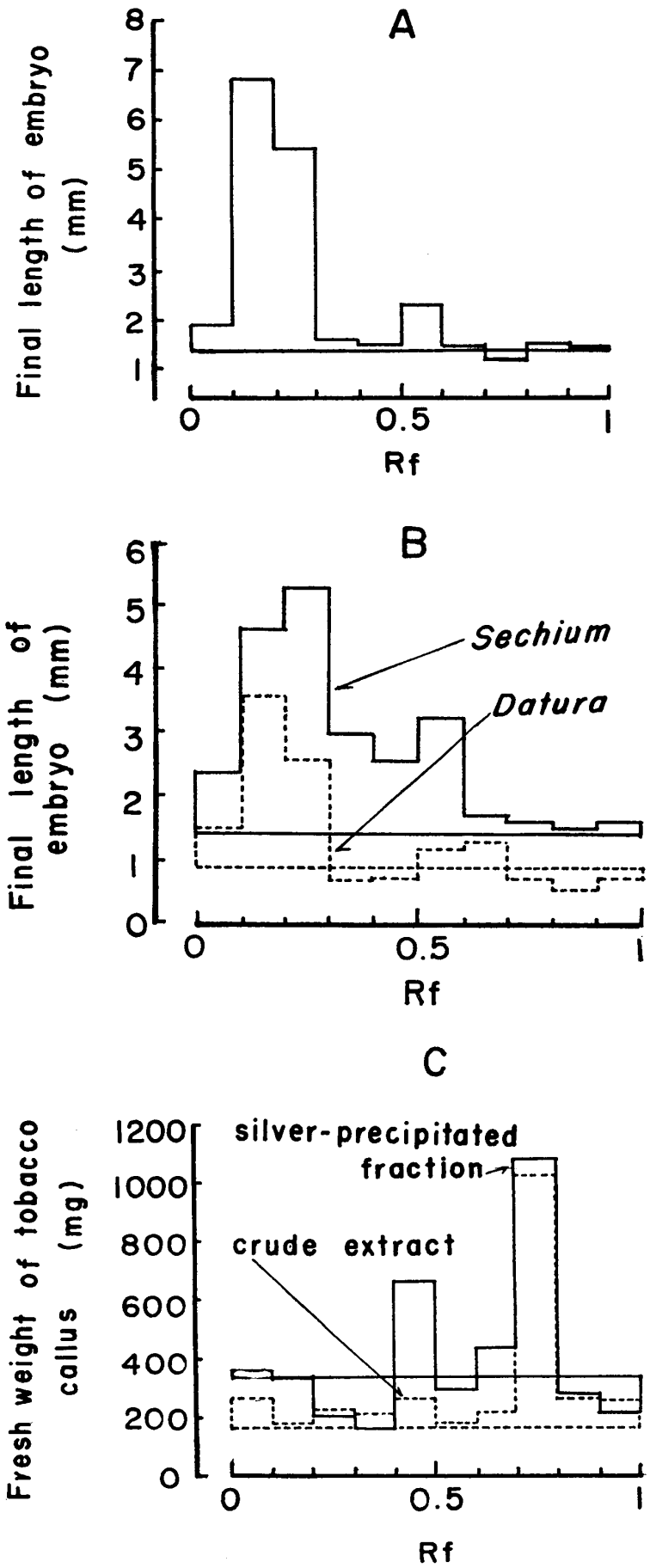
Paper chromatopraphy : In a previous paper it was reported that ethanol extracts of young *Datura* seeds and of old *Sechium* embryos also contained embryo factors[11]. Therefore in this experiment Fraction 6 obtained above and these *Datura* and *Sechium* extracts as well as the silver-precipitated fraction were studied with paper chromatography. Ten GE of materials were streaked at the foot of Toyo filter paper No. 51 separately and were developed ascendingly over 20 cm as bands with a solvent system : *n*-butanol-acetic acid-water (4 : 1 : 2, v/v). Chromatograms were dried and divided crosswise into ten zones in accordance with Rf value. Every zone was eluted with 30 ml of water. Each eluate was added to the basal medium and tested at a reasonable level for their growth-promoting activity. The histograms showing embryo factor activities were shown in Fig. 5 (A, B).

Active substance (or substances) in Fraction 6 was distributed in Rf 0.1-0.3. (Fig. 5A). A slight activity was found at Rf 0.5 -0.6. In *Datura* and *Sechium* extracts similar histograms were also obtained (Fig. 5 B). However in *Sechium* the largest activity was at Rf 0.2-0.3 and a moderate activity at Rf 0.5-0.6. These results indicates that the same or similar embryo factors are present in *Lupinus, Datura,* and *Sechium* seeds.

The crude *Lupinus* extract and the silver-precipitated fraction were also developed likewise to study cytokinin. Chromatogram of silver-precipitated fraction showed an active peak at Rf 0.7-0.8 and another weakly active one at Rf 0.4-0.5 (Fig. 5C). Similar peaks with the highest at Rf 0.7-0.8 were obtained from the crude extract. The correspondence of Rf values of the peak between the crude extract and the silver-precipitated fraction seems to indicate that cytokinin in the latter is not an artifact produced during chemical procedures.

In a separate experiment it was shown that the Rf values of kinetin, 6-benzylamino purine, and adenine developed with the same solvent were 0.88, 0.92, and 0.52, respectively, indicating that the cytokinin in *Lupinus* seed was different from these substances.

As shown in Fig. 5, the Rf value of embryo factor was much lower than that of cytokinin. Therefore two activities in the same extract were attributed to quite different substances. This conclusion may be compatible with results of the investigations in isolated embryos and cultured tissues. Kinetin, which stimulates the cell division of cultured tissues of tobacco and carrot, never promoted the growth of heart-shaped embryos[19]. On the other hand casein hydrolysate, which



was promotive considerably to the embryo growth[19], did not act as cytokinin in tissue culture.

Nevertheless young seeds and liquid endosperm are the richest sources of these factors. This suggests that cytokinin also may have some physiological roles in the seed and embryo growth. It may act on different stages of young embryo. Embryo factor was promotive most efficiently to the growth of globular and heart-shaped embryos, but it has not been proved to stimulate still younger embryos. Cytokinin may promote the growth of undifferentiated cells shortly after fertilization, because it stimulates the division of dedifferentiated cells remarkably.

A previous paper reported that cytokinin in *Lupinus* seed was a purine derivative according to its color reaction with silver nitrate-bromophenol blue reagent[20]. On the other hand chemical properties of embryo factor have been still unknown. Purification of these factors and the studies on physiological roles of them in seed development should be advanced more in detail.

Fig. 5. Histograms showing the presence of embryo factor in Fraction 6 (A) and in *Datura* and *Sechium* extracts (B), and of cytokinin (C) in the crude extract and in the silver-precipitated fraction. Paper chromatograms were developed with a solvent system: *n*-butanol-acetic acid-H_2O (4 : 1 : 2).

Summary

1. The present study is concerned with cytokinin and embryo factor in young *Lupinus* seeds.

2. Partial purification of these factors was carried out.

3. The silver-precipitated fraction of the *Lupinus* extract stimulates cell-division of calluses of tobacco and carrot. It is promotive to the growth of radish leaf, and inhibitory to the degradation of chlorophyll of radish cotyledon.

4. Embryo factor and cytokinin in the *Lupinus* extract have different Rf values on paper chromatogram, indicating that they were different substances.

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