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Analysis of polyamine metabolism in soybean seedlings using ^{15}N-labelled putrescine

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Abstract

The translocation and metabolism of polyamines during soybean germination were studied using ^{15}N-labelled putrescine as a precursor. Both ^{15}N-labelled and unlabelled polyamines were simultaneously detected using a novel application of ionspray ionization-mass spectrometry. ^{15}N-putrescine was rapidly transported to the shoots and roots, where it was converted to spermidine and spermine. The main ^{15}N-polyamine that accumulated in the root was ^{15}N-spermine. It was found that there were differences in the way endogenous putrescine and exogenous ^{15}N-putrescine were metabolized in soybean seedlings.

Keywords: *Glycine max*; Leguminosae; Soybean; Metabolism; Translocation; Polyamine

1. Introduction

Aliphatic polyamines, which are ubiquitous compounds that are classified as growth factors (Bagni, 1989), have been implicated in a number of plant growth and developmental processes, including cell division (Bagni, 1989), embryogenesis, morphogenesis (Bagni et al., 1993) and responses to environmental stresses (Bouchereau et al., 1999). Plant hormones and light are important in the control of plant growth, development and polyamine metabolism (Caffaro et al., 1994).

In order to understand the role of polyamines in plants, not only the study of their biosynthesis but also that of long-distance transport are essential. High polyamine concentrations and high activity levels of polyamine biosynthetic enzymes are closely associated with rapid cell division in plants as well as in other organisms (Galston and Kaur-Sawhney, 1990; Walden et al., 1997). Marked changes in polyamine concentrations have been observed during germination of soybean (*Glycine max* (L.) Meer; cv. Williams), a short-day plant that is widely used in photoperiodism studies (Scoccianti et al., 1990). Intracellular concentrations of polyamines can be regulated both by translocation and biosynthesis (Friedman et al., 1986).

Radioactive compounds such as ^{14}C-spermidine (Caffaro et al., 1994) have previously been utilized to study the translocation of polyamines in adult soybean plants. However, this approach does not always reveal intracellular turnover and translocation of polyamines. Therefore, in our present study, attempts were made to investigate the uptake of ^{15}N-labelled putrescine and its conversion to spermidine and spermine during soybean germination with a new method for simultaneous determination of ^{15}N-labelled and non-labelled polyamines by ionspray ionization-mass spectrometry

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(Furuumi et al., 1998). This method was first applied to animal tissues (Xu et al., 2002). It is unique in that it can separately measure the absolute amounts of both endogenous and exogenous polyamines. The aim of the present study was to investigate the metabolism of exogenous ^{15}N-putrescine in different organs of soybean plants that were grown under light or dark conditions.

2. Results

Soybean seeds were first soaked in ^{15}N-putrescine solution for 18 h. Ionspray ionization-mass spectrometry was then used to determine the absolute amount of ^{15}N-putrescine, ^{14}N-putrescine, ^{15}N-spermidine, ^{14}N-spermidine, ^{15}N-spermine and ^{14}N-spermine in the embryo, cotyledon and other plant organs (Table 1). The polyamine concentrations were expressed as nmol per gram of fresh weight (nmol g^{-1} FW).

Spermidine is generally the most abundant polyamine present in plant embryos, followed by putrescine and spermine (Scoccianti et al., 1990). In the soybean seed cotyledon, both spermidine and spermine are found in significant amounts, whereas the putrescine concentrations are quite low. At the start of germination there was an increase in the amount of all the non-labelled polyamines especially spermidine in the shoot. Similar patterns were observed in the root, whereas spermidine and spermine levels in the cotyledon decreased by day 4. Polyamine concentrations reached their maximum in the shoots at 6 days after germination, whereas putrescine and spermidine levels had decreased by day 8 (Scoccianti et al., 1990). Similar results were obtained from the seeds that were kept under dark conditions (Table 1).

The level of ^{15}N-spermine in the embryo was much higher than that of ^{15}N-putrescine or ^{15}N-spermidine. The level of ^{15}N-spermidine and ^{15}N-spermine in the cotyledon were much lower than that of ^{15}N-putrescine. The percentage of ^{15}N-polyamine in each organ was calculated as (^{15}N-polyamine/^{15}N-polyamine + ^{14}N-polyamine) × 100 (Fig. 1).

Table 1
Endogenous ^{14}N-polyamine and ^{15}N-labelled polyamine contents (nmol g^{-1} FW) in embryos, cotyledons and other plant organs during germination

	^{14}N-put	^{15}N-Put	^{14}N-Spd	^{15}N-Spd	^{14}N-Spm	^{15}N-Spm
Seed						
Embryo	56.5	7.2	69.6	8.7	15.9	214.5
Cotyledon	1.0	7.2	15.8	0.8	17.6	1.4
Light						
Seedling						
4 day						
Shoot	60.3	15.5	153.4	32.8	43.1	79.3
Cotyledon	1.4	0.6	11.9	4.0	7.2	1.5
Root	24.0	3.2	74.1	11.1	14.1	185.9
6 day						
Shoot	124.2	12.1	169.7	21.2	54.5	330.3
Cotyledon	1.5	0.3	17.7	3.9	5.3	2.5
Root	6.6	0.8	18.5	2.5	3.5	132.8
8 day						
Shoot	24.2	4.2	78.9	6.3	75.8	23.2
Cotyledon	0.9	0.1	12.4	2.1	5.4	2.0
Hypocotyl	2.5	0.4	8.8	1.0	3.6	18.5
Root	8.7	0.5	15.1	1.3	1.3	222.8
Dark						
Seedling						
4 day						
Shoot	20.0	10.0	103.3	20.0	26.7	16.7
Cotyledon	3.3	2.5	10.6	3.1	6.2	2.2
Root	22.0	3.5	50.2	8.7	10.4	457.8
6 day						
Shoot	153.8	30.8	307.7	46.2	69.2	107.7
Cotyledon	2.5	0.6	20.3	4.4	5.0	8.7
Root	12.8	1.1	18.9	2.8	2.9	268.7
8 day						
Shoot	95.0	15.0	195.0	20.0	75.0	20.0
Cotyledon	2.1	0.4	28.7	5.6	8.5	3.4
Hypocotyl	7.7	0.6	9.7	1.2	2.6	95.4
Root	13.4	1.2	14.9	1.5	1.5	212.6

Put, putrescine; Spd, spermidine; Spm, spermine.

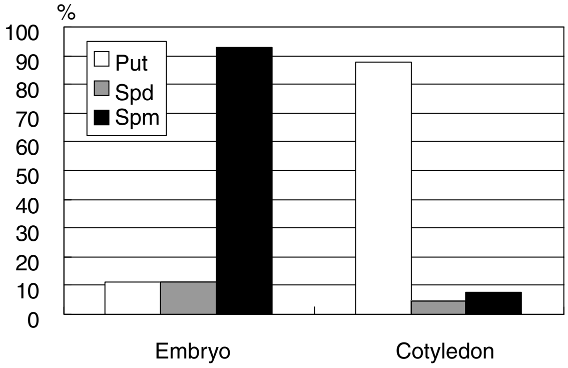

Fig. 1. The distribution of ^{15}N-polyamines in the embryos and cotyledons after exposure to ^{15}N-putrescine for 18 h.

Under light conditions, the percentage of ^{15}N-spermine in the root was much greater than that of ^{15}N-putrescine and ^{15}N-spermidine at both 4 and 6 days after germination, and reached its maximum value at day 8. By contrast, the percentage of ^{15}N-spermine in the cotyledon and shoot peaked at 6 days after germination and had decreased by day 8. In the hypocotyl, the percentage of ^{15}N-spermine was much higher than that of both ^{15}N-spermidine and ^{15}N-putrescine at day 8. The percentage of ^{15}N-spermidine gradually decreased over time in both the cotyledon and the shoot, whereas it remained relatively constant in the root throughout germination (Fig. 2).

Under dark conditions, the percentage of ^{15}N-spermine in the root reached its maximum at 4 days after germination and subsequently remained high, with no marked changes in ^{15}N-spermidine levels. The percentage of ^{15}N-spermine in the cotyledon and shoot was lower than in soybean plants grown in the light. However, the percentage of ^{15}N-spermine in the hypocotyl at day 8 after germination was greater than that observed under light conditions (Fig. 3). In both the cotyledon and shoot, the percentage of ^{15}N-putrescine was elevated at day 4. In short, essentially the same changes in polyamine levels were observed during germination under both light and dark conditions.

3. Discussion

The results of our present study have shown that ^{15}N-putrescine is taken up by soybean seeds and rapidly converted to ^{15}N-spermidine and ^{15}N-spermine. Large amounts of ^{15}N-spermine were synthesized from ^{15}N-putrescine and accumulated in the root and shoot, even though the concentrations of endogenous non-labelled spermine were quite low. The discrepancy can be explained at least partially by the fact that endogenous putrescine is synthesized not only from ornithine and but also from arginine via polyamine biosynthetic enzymes in soybean plants.

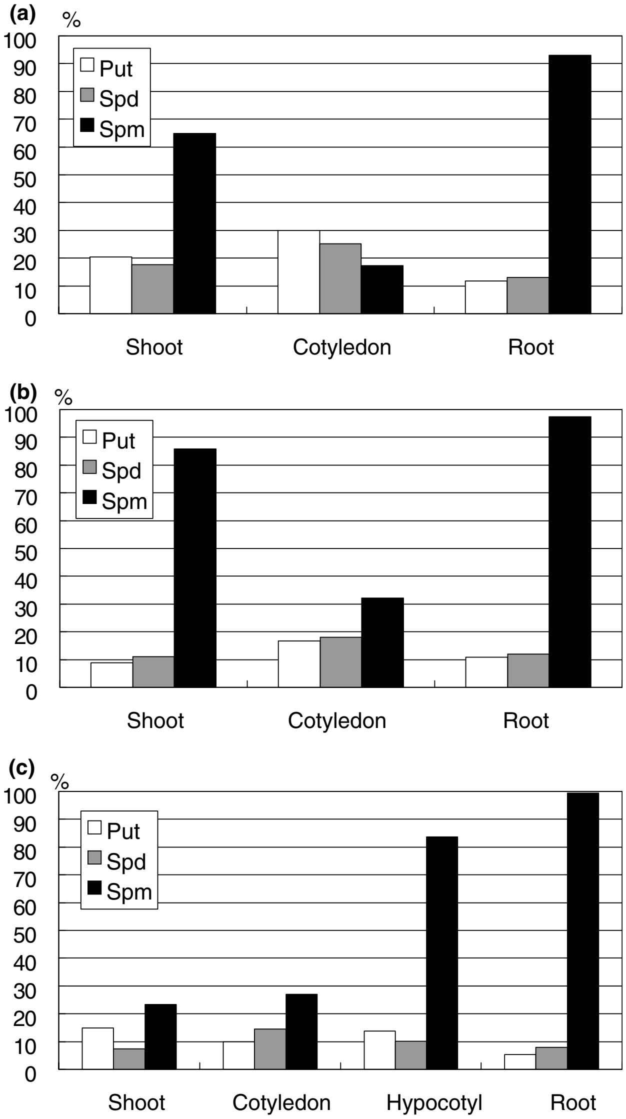

Fig. 2. The distribution of ^{15}N-polyamines in different organs during germination under light conditions: A, day 4; B, day 6; C, day 8.

Initial polyamine uptake may possibly be the result of diffusion across a concentration gradient. The translocation of polyamines generally occurs from the cotyledon to the embryo during the first 18 h of germination (Lin, 1984). In tomato and corn seedlings, ^{3}H-putrescine is absorbed by roots against a concentration gradient and is translocated to the upper parts of the seedling within 30 min (Rabiti et al., 1989). A certain proportion of the absorbed putrescine is converted to spermidine and spermine in these plants. In addition, ^{14}C-spermidine is translocated from the leaves to all parts of soybean plants (Caffaro et al., 1994). However, precise measurement of polyamine levels is quite difficult in experiments with radioactive compounds. Our present method was able to measure the absolute amounts of ^{15}N-spermidine and ^{15}N-spermine, both of which were derived from exogenous ^{15}N-putrescine.

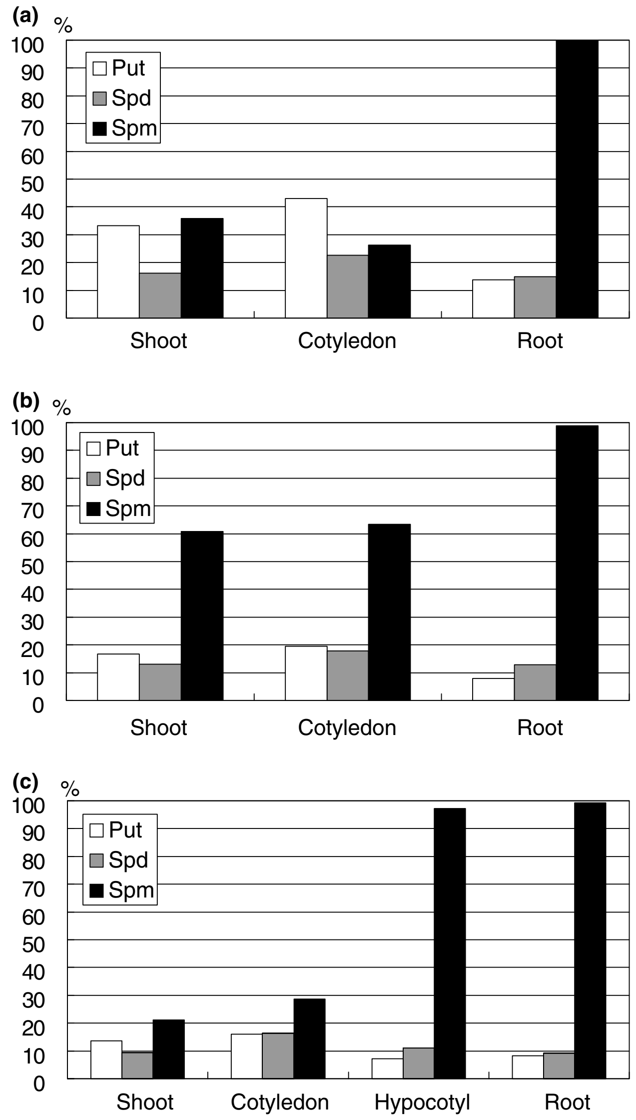

Fig. 3. The distribution of ^{15}N-polyamines in different organs during germination under darkness: A, day 4; B, day 6; C, day 8.

The percentage of ^{15}N-putrescine in the cotyledon was much higher than in the shoot and root at the early stages of germination, and decreased gradually over time (Figs. 2 and 3). This indicates that the conversion of putrescine to spermidine and then to spermine is relatively slow in the cotyledon, and that ^{15}N-putrescine is rapidly transported to the shoot and root. Lin (1984) reported that putrescine in the soybean cotyledon is translocated to the embryo. Experiments with cotyledons grown in vitro under light conditions have revealed that the capacity to synthesize spermidine increases with age (Bagni, 1970).

The observation that the percentage of ^{15}N-spermine in the root is high throughout germination indicates that ^{15}N-spermine is continuously synthesized and accumulated in this organ. Eight days post-germination, the percentage of ^{15}N-spermine was lower in the shoot and hypocotyl than in the root (Figs. 2 and 3). This suggests a gradient in the percentage of ^{15}N-spermine in the root through the hypocotyl to the shoot as a result of translocation. Caffaro et al. (1993) showed that in the soybean, ^{14}C-spermidine administered through the leaf formed a gradient along the plant axis by both basipetal and acropetal translocation. The fact that the percentage of ^{15}N-putrescine in the cotyledon was much higher than in the shoot and root at the early stages of germination suggests that ^{15}N-putrescine is transported from the cotyledon to these other organs.

The percentage of ^{15}N-spermine in the hypocotyl at day 8 was much higher than that of either ^{15}N-spermidine or ^{15}N-putrescine (Figs. 2 and 3). In addition, the percentage of ^{15}N-spermine in the hypocotyl of soybeans grown under dark conditions was greater than that of soybeans grown in the light. This might be the result of differences in hypocotyl length, as hypocotyls of seedlings that were germinated in darkness extended more rapidly than those that were grown under light conditions. Smith and Davies (1985) reported that polyamines in the hypocotyl increase during internode elongation, probably as a result of initial cell proliferation.

Only a small amount of the ^{15}N-putrescine present in the cotyledon was metabolized, as shown in Table 1 and the figures. Torrigiani and Scoccianti (1995) reported that arginine decarboxylase activity in the embryo was higher than that in the cotyledon in chick-pea seeds. They also reported that arginine and ornithine decarboxylase activities in shoots, and particularly in roots, were higher than those in cotyledons of chick-pea seedlings. Taken together, these results suggest that greater levels of polyamine synthesis and metabolism might occur in embryos, shoots and roots than in cotyledons. Therefore, the cotyledon may function mainly in transferring polyamines to other organs.

Using ionspray ionization-mass spectrometry technique, we previously succeeded in assaying endogenous non-labelled polyamines and exogenous ^{15}N-putrescine and ^{15}N-polyamines at the same time in living animals (Xu et al., 2002; Kobayashi et al., 2003). Our present results revealed that there were differences in the way endogenous putrescine and exogenous ^{15}N-putrescine were metabolized in soybean seedlings. The findings suggest that external feeding of ^{15}N-putrescine does not necessarily reflect the in vivo situation and limit the usefulness of the approaches to identify the biological fates and roles of polyamines in soybean seedlings. It is likely that ^{15}N-putrescine that is transferred to the shoot and root is used to synthesize ^{15}N-spermidine, which is then rapidly converted to ^{15}N-spermine. Endogenous ^{14}N-putrescine that is synthesized locally may be metabolized differently from ^{15}N-putrescine, possibly because it is present in different compartments: both compounds must be converted to other polyamines in a similar manner if they are present in the same compartment. It was an unexpected finding that a large amount of ^{15}N-spermine was accumulated in the soybean root and shoot. These findings suggest either that ^{15}N-putrescine is metabolized differently according to its location, or that ^{15}N-spermine is more resistant than ^{14}N-spermine to polyamine-metabolizing enzymes, such as oxidases because of the difference in their molecular mass. We previously reported that activities of ornithine and arginine decarboxylase in *Nicotiana glauca* leaves occur at different locations: the chloroplasts and cytosol, respectively (Bagni et al., 1986). In addition, diamine oxidase activity is mainly located in the root (Scoccianti et al., 1990) and its substrate specificity depends on the number of amino groups and CH^2 bonds in the polyamine molecule in soybean seedlings (Suzuki, 1973).

Other explanations for these results cannot be excluded. Because the concentration of ^{15}N-putrescine in the shoot and root transported from the cotyledon is expected to be much higher than that of endogenous putrescine, ^{15}N-spermidine might be preferentially synthesized and then rapidly converted to ^{15}N-spermine. On the other hand, only a small amount of endogenous ^{14}N-spermine can be produced, because endogenous putrescine is maintained in physiological relative low concentrations. Because putrescine is synthesized not only from ornithine but also from arginine in plants, the availability of L-arginine derived from breakdown of proteins in the cotyledon must be an important factor for the determination of putrescine synthesis in the shoot and root of soybean seedlings.

In conclusion, our study shows that exogenous ^{15}N-putrescine is taken up in soybean cotyledons and transported to various other organs in which it is used as a substrate for polyamine synthesis. Not only the activity of polyamine biosynthetic enzymes but also the translocation of precursors would regulate the metabolism of polyamines in soybean.

4. Experimental

4.1. Chemicals

[1,4-$^{15}N_2$]-putrescine (^{15}N-putrescine) · 2HCl, [1,4-$^{13}C_2$, 1,4-$^{15}N_2$]-putrescine (^{13}C,^{15}N-putrescine) · 2HCl, [5,8-$^{13}C_2$, 1,4,8-$^{15}N_3$]-spermidine (^{13}C,^{15}N-spermidine) · 3HCl and [5,8-$^{13}C_2$,1,4,8,12-$^{15}N_4$]-spermine (^{13}C,^{15}N-spermine) · 4HCl were prepared according to previously reported methods (Xu et al., 2002). ^{15}N-putrescine was used for the treatment of soybean. ^{13}C,^{15}N-putrescine, ^{13}C,^{15}N-spermidine and ^{13}C,^{15}N-spermine served as internal standards for polyamine determination by ionspray ionization-mass spectrometry.

4.2. Plant material and treatment of soybean with ^{15}N-putrescine

Soybean (*Glycine max* (L.) Meer; cv. Sakai) seeds, which were harvested in 2002, were soaked in 0.2 mM ^{15}N-labelled putrescine hydrochloride solution for 18 h. They were then divided into two groups and grown in wet-soil pots for 8 days. One group was allowed to germinate in a greenhouse with a photoperiod of 12 h natural light/12 h darkness and a day/night temperature regime of 28/16 °C, respectively. The other group was kept in darkness. On day 8, the hypocotyl emerged and the shoots became green under light conditions. After harvest, the seedling organs were separated for polyamine analysis. Each sample was homogenized in 3 volumes of 5% trichloroacetic acid and centrifuged for 15 min at 1500*g*.

4.3. Polyamine determination by ionspray ionization-mass spectrometry

An API 300 mass spectrometer (PE-SCIEX, Thornhill, Canada) with an attached ionspray-ionization interface (IS-MS) was used to determine polyamine levels according to the previously reported method of Furumi et al. (1998). Samples that were subjected to heptafluorobutyryl derivatization were dissolved in 50% acetonitrile–water containing 0.5% ammonium acetate. Sample injection was carried out using a Rheodyne bulb (loop size 5 μl) set between the ionspray probe and the syringe drive (Harvard Apparatus 22), which pumped 50% $CH_3CN–H_2O$ containing 0.5% ammonium acetate at a constant flow rate of 10 μl/min. The mass ranges for ions to be integrated were set as described previously (Xu et al., 2002).

All experiments were repeated twice and provided similar results. Therefore, the data presented refers to a single experiment.

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