Polyamines in Different Organs of \textit{Brassica} Crop Plants with or without Clubroot Disease

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Abstract: Polyamines acid extracted from roots, stems, leaves, flower buds, flowers and seeds of eight \textit{Brassica} crop plants (cabbage, broccoli, cauliflower, Komatsuna, Chingensai, turnip, Aburana and Seiyou-aburana) with or without clubroot disease were analyzed by high-performance liquid chromatography and gas chromatography. Endogenous concentrations of polyamines per wet weight of the organ were calculated. In cabbage, broccoli and Komatsuna clubroot galls, the levels of putrescine, spermidine and spermine were increased whereas the levels of agmatine levels were decreased after the infection with a protist, \textit{Plasmodiophora brassicae}. The levels of 2-phenylethylamine and homospermidine found in the normal healthy roots were decreased in the clubroots. The thermospermine level in broccoli was higher in the sprout stem than in the sprout root or other organs. A high agmatine level was found in the healthy sprouts and flower buds of broccoli and cauliflower flower buds. Diaminopropane, cadaverine and norspermine were detected in some \textit{Brassica} organs as a minor polyamine. The seeds of the eight \textit{Brassica} plants were rich in spermine and spermidine.

Key words: Agmatine, \textit{Brassica} crop plant, Clubroot, Homospermidine, Polyamine, Thermospermine.

Among the biogenic amines found to act as endogenous polyamines in higher land plants (Miuguet et al., 2008; Fuell et al., 2010; Takano et al., 2012), tetra-amines (e.g. spermine, thermospermine and norspermine) and some penta-amines play a defensive role against thermal and dry stresses in the seeds of leguminous and gramineous crop plants (Hamana and Matsuzaki, 1993; Hamana et al., 1992, 1994, 1996), and against osmotic stress in aquatic plants (Hamana et al., 1994, 1998, 2000). After the sprouting of seeds, leguminous and gramineous seedlings showed an increase in diamines such as diaminopropane, putrescine, cadaverine and agmatine, a guanidinoamine as a source of long polyamine synthases (Hamana and Matsuzaki, 1993; Hamana et al., 1994, 1996), so that these polyamines are involved in plant cell proliferation and differentiation (Kusano et al., 2008; Takahashi and Kakehi, 2010).

The clubroot (root gall) disease occurring in the family \textit{Brassicaceae} (formerly \textit{Cruciferae}) is caused by the obligate biotrophic protist \textit{Plasmodiophora brassicae} and is one of the economically important diseases of \textit{Brassica} crop plants. A model plant, \textit{Arabidopsis thaliana}, belonging to the family \textit{Brassicaceae}, which has lost ornithine decarboxylase for synthesis of putrescine and produces putrescine from agmatine. Therefore, polyamine synthases of \textit{Brassica} plants might depend on arginine decarboxylase activity (Hanfrey et al., 2001; Jubault et al., 2008). When agmatine accumulated in response to the infection of \textit{P. brassicae} to \textit{A. thaliana} roots, putrescine, spermidine and spermine levels were not significantly altered (Hanfrey et al., 2001). In a turnip, \textit{Brassica rapa} ver. \textit{rapa}, the levels of putrescine, spermidine and spermine have been reported to be higher in the roots infected with clubroot than in the non-infected normal roots; however, the level of agmatine has not been reported (Walters and Shuttleton, 1985; Walters 2003).

In the present study, we examined the endogenous levels of polyamines including 2-phenylethylamine (an aromatic amine produced from \textit{L}-phenylalanine), agmatine and unusual polyamines such as homospermidine, norspermidine, thermospermine and norspermine in various organs of three \textit{Brassica} crop plants, cabbage, broccoli and Japanese mustard spinach (Komatsuna). The effects of clubroot on polyamines in mature leaves and roots were also examined. We also analyzed the polyamines in the leaves, flower buds, flowers, sprouts and/or seeds of eight species of \textit{Brassica} crop plants to obtain not only plant-physiological data but also nutritional information of polyamines.
Materials and Methods

1. Brassica plant materials

Young healthy plants (20 – 30 days after sowing, DAS) of three Brassica plants (Brassica oleracea var. cymosa) flower buds, cauliflower flower buds, Romanesco (B. oleracea var. botrytis L. botrytis) flower buds, rapeseed (Aburana) flower buds, qing geng cai (Chingensai) leaves, and turnip roots and leaves (cultivar name unknown), were purchased from Maebashi Nursery Co., Maebashi, Gunma, Japan. A soil fungicide containing fensulphamide (Nebijin; Kumiai Chemical Industry Co., Ltd., Tokyo, Japan), a soil improvement agent containing calcium silicate (Minecal; The Sangyo Shinko Co., Ltd., Tokyo) and a magnesium fertilizer (New-Ecomag; Naikai Shoji Co., Ltd., Tokyo) were used to inhibit the clubroot disease.

The young plants were cultivated in soil contaminated with P. brassicae in the experimental field of Kuroiwa Survey Design Office Co., Maebashi, either with or without supplementation of Minecal (10%(w/w)), New-Ecomag (2.0 g L⁻¹) and Nebijin (0.25 g L⁻¹). No galls were formed on the roots of the mature plants in the plots with the supplementation, which indicated that clubroot disease did not occur. These plants are referred to as mature healthy plants hereafter. The roots and leaves of the young healthy plants before planting (20 – 30 DAS), the roots and leaves of the mature healthy plants (120 – 150 DAS), the roots with clubroot galls (CB roots) in the plots without the supplementation, the normal roots of the plants (normal roots) in the plots without the supplementation and the leaves of the plants (120 – 150 DAS) (infected leaves) in the plots without the supplementation were harvested. Broccoli flower buds were harvested from the mature healthy plants.

Broccoli sprouts were purchased from Fueki Farm, Niigata, Japan and Murakami Farm, Chiba, Japan. Sticksenor (a hybrid of broccoli, B. oleracea conv. botrytis var. cymosa) flower buds, cauliflower flower buds, Romanesco (a cultivar of cauliflower, B. oleracea var. botrytis I. botrytis) flower buds, rapeseed (Aburana) flower buds, qing geng cai (Chingensai) leaves, and turnip roots and leaves (cultivar name unknown), were purchased in Maebashi. Seeds of cabbage (“Tokinashi”), broccoli (“Green Palace”), Komatsuna (“Chusei Komatsuna”), cauliflower (“Hakusen”), Aburana (“Subomina”), Chingensai (“Chingensai”) and turnip (“Tokinashi-kokabu”) were purchased from Atariya Farm Co., Chiba or Utane Seed Co., Utsunomiya, Japan. Seeds of the rapeseed (Seiyounaburana) (“Nahana”) purchased from Tohoku Seed Co., Utsunomiya, were cultivated in the garden of Koei Hamana, Maebashi. Roots and leaves of the young healthy plants (30 DAS), and flower buds and flowers of the mature healthy plants (150 DAS) were harvested.

2. Polyamine analysis

Plant organs (10 – 100 g) were homogenized in the same weight of 10% (1.0 M) perchloric acid (PCA) with a mixer. After extraction with 5% PCA three times, the supernatant of the PCA extract was applied to a column of a cation-exchange resin, Dowex 50Wx8, and then polyamines were eluted with 6 M HCl from the column (Hamana and Matsuzaki, 1993; Hamana et al., 1992, 1994, 1996, 1998, 2000).

The concentrated polyamines were analyzed by high-performance liquid chromatography (HPLC) on a Hitachi L6000 using a column of cation-exchange resin (Hitachi 2619F, 4 mm I.D. × 50 mm) at 70°C and determined by post-labeled fluorometry after heating with 2-mercaptoethanol (Hama et al., 1995; Hamana, 2002). After heat-stabilization of the concentrated polyamines, gas chromatography (GC) using a capillary column of Inert Cap 1MS (0.32 mm I.D. × 30 m) (GL Sciences) was performed on a SHIMADZU GC-17A or JEOL JMS-700 equipped with a flame-ionization detector, at the column of temperature 120°C(90°C)-16°C/min-280°C (Niitsu et al., 1993, 2014; Otsuka et al., 2007). 2-Phenylethylamine, diaminopropane, putrescine, cadaverine, norspermidine, spermidine, norspermine, spermine and agmatine, purchased from Sigma (St. Louis, USA) or Eastman Kodak (Rochester, USA), and homospermidine and therspermine, synthesized in our laboratory, were used as the standards. Molar concentrations of polyamines per gram of wet weight of the starting organ sample (μmol/g w.w.) calculated from the HPLC and GC analyses are shown in Table 1.

Results and Discussion

1. Polyamine profiles in Brassica roots with and without clubroot

Polyamine concentrations in the extract from plants in the plots with the supplementation (mature healthy roots), the roots with clubroot galls in the infected plots without the supplementation (CB roots), the normal roots of the infected roots without the supplementation (normal roots), and young roots (before planting) in three Brassica plants are shown in Table 1. In cabbage, putrescine and spermidine levels were higher in the CB roots, 0.22 and 2.00 μmol/g w.w., respectively than in the young roots, 0.19 and 0.63 μmol/g w.w., respectively, and the mature healthy roots, 0.07 and 0.70 μmol/g w.w., respectively. The spermidine level in the CB roots was twofold that in the normal roots. The spermine level increased from 0.02 – 0.03 to 0.06 μmol/g w.w. in the CB roots. The concentration of agmatine in the normal roots, young roots, mature healthy roots and CB roots was 0.29, 0.47, 0.28 and 0.10 μmol/g w.w., respectively. 2-Phenylethylamine appeared in the normal roots and mature healthy roots, but the level was even lower in the CB roots and young roots.

In broccoli, the spermidine level in the CB roots (1.41 μmol/g w.w.) was twofold that in the normal roots (0.77
Table 1. Polyamine concentrations in eight Brassica crop plants.

| Organs of Brassica plants | Pea | Dap | Put | Spd | HSpd | TSpm | Spm | Agm |
|---------------------------|-----|-----|-----|-----|------|------|-----|-----|
| Brassica oleracea var. capitata (cabbage) | | | | | | | | |
| Root with clubroot gall [2] | 0.03 | ND | 0.22 | 2.00 | 0.03 | 0.02 | 0.06 | 0.10 |
| Normal root without clubroot [2] | 0.06 | ND | 0.09 | 0.93 | 0.02 | 0.02 | 0.03 | 0.29 |
| Mature healthy root [2] | 0.04 | ND | 0.07 | 0.70 | ND | 0.02 | ND | 0.28 |
| Young root [2] | 0.01 | ND | 0.19 | 0.63 | 0.07 | 0.02 | 0.21 | 0.47 |
| Young leaf [2] | 0.01 | ND | 0.04 | 2.29 | ND | 0.01 | 0.02 | 0.31 |
| Mature leaf [2] | ND | ND | 0.64 | 1.33 | ND | 0.02 | 0.02 | 0.16 |
| Mature leaf of infected plant [1] | ND | ND | 0.30 | 1.18 | ND | 0.02 | 0.01 | 0.10 |
| Seed [1] | ND | ND | 0.06 | 2.40 | ND | 0.04 | ND | 1.00 |

| Brassica oleracea var. italica (broccoli) | | | | | | | | |
| Root with clubroot gall [3] | 0.01 ± 0.01 | ND | 0.07 ± 0.03 | 1.41 ± 0.11 | 0.02 ± 0.01 | 0.01 ± 0.01 | 0.08 ± 0.01 | 0.03 ± 0.01 |
| Normal root without clubroot [3] | 0.10 ± 0.02 | ND | 0.05 ± 0.03 | 0.77 ± 0.12 | 0.07 ± 0.02 | 0.02 ± 0.00 | 0.10 ± 0.05 | 0.10 ± 0.06 |
| Mature healthy root [3] | 0.05 ± 0.02 | ND | 0.03 ± 0.02 | 0.63 ± 0.19 | 0.03 ± 0.01 | 0.01 ± 0.01 | 0.12 ± 0.03 | 0.25 ± 0.09 |
| Young root [3] | 0.07 ± 0.02 | ND | 0.12 ± 0.02 | 1.30 ± 0.08 | 0.05 ± 0.02 | 0.01 ± 0.01 | 0.07 ± 0.02 | 0.26 ± 0.05 |
| Sprout root [3] | ND | 0.02 ± 0.00 | 0.88 ± 0.36 | 1.78 ± 0.13 | 0.11 ± 0.05 | 0.02 ± 0.01 | 0.15 ± 0.05 | 0.98 ± 0.23 |
| Sprout stem [4] | ND | 0.02 ± 0.02 | 1.35 ± 0.66 | 0.97 ± 0.36 | 0.05 ± 0.04 | 0.09 ± 0.04 | 0.11 ± 0.05 | 3.26 ± 0.57 |
| Young leaf [3] | ND | ND | 0.17 ± 0.08 | 1.45 ± 0.17 | 0.02 ± 0.01 | 0.04 ± 0.01 | 0.08 ± 0.02 | 0.20 ± 0.08 |
| Mature leaf [3] | 0.02 ± 0.02 | ND | 0.27 ± 0.13 | 1.46 ± 0.19 | ND | 0.02 ± 0.00 | 0.17 ± 0.06 | 0.35 ± 0.07 |
| Mature leaf of infected plant [1] | ND | ND | 0.10 | 1.36 | ND | 0.02 | ND | 0.24 |
| Old leaf [3] | ND | ND | 0.09 ± 0.05 | 1.10 ± 0.25 | ND | 0.02 ± 0.01 | 0.10 ± 0.03 | 0.09 ± 0.06 |
| Flower bud [3] | 0.01 ± 0.02 | 0.02 ± 0.02 | 0.36 ± 0.16 | 1.57 ± 0.39 | ND | 0.05 ± 0.02 | 0.22 ± 0.11 | 2.82 ± 0.35 |
| Flower bud (Sticksensor) [3] | ND | 0.02 ± 0.02 | 0.88 ± 0.36 | 1.78 ± 0.13 | 0.11 ± 0.05 | 0.02 ± 0.01 | 0.15 ± 0.05 | 0.98 ± 0.23 |
| Seed [1] | ND | ND | 0.08 | 2.40 | ND | ND | 1.26 | 0.30 |

| Brassica oleracea var. botrytis (cauliflower) | | | | | | | | |
| Flower bud [3] | 0.02 ± 0.02 | 0.06 ± 0.04 | 0.38 ± 0.15 | 1.62 ± 0.33 | 0.01 ± 0.01 | 0.08 ± 0.02 | 0.42 ± 0.09 | 0.31 ± 0.07 |
| Flower bud (Romanesco) [3] | ND | 0.03 ± 0.01 | 0.22 ± 0.05 | 1.24 ± 0.38 | ND | 0.08 ± 0.02 | 0.23 ± 0.05 | 1.20 ± 0.14 |
| Seed [1] | ND | ND | 0.10 | 2.80 | ND | ND | 0.80 | 0.28 |

| Brassica oleracea var. b. var. p. (Japanese mustard spinach) | | | | | | | | |
| Matve leaf [3] | ND | 0.07 ± 0.02 | 0.22 ± 0.07 | 1.12 ± 0.05 | 0.01 ± 0.01 | ND | 0.03 ± 0.01 | 0.02 ± 0.01 |
| Seed [1] | ND | ND | 0.10 | 1.50 | ND | ND | 0.10 | 0.04 |

| Brassica napus var. n. (rapeseed) | | | | | | | | |
| Flower bud [3] | ND | 0.05 ± 0.06 | 0.33 ± 0.08 | 2.17 ± 0.31 | ND | 0.03 ± 0.00 | 0.18 ± 0.08 | 0.08 ± 0.02 |
| Seed [1] | ND | ND | 0.40 | 2.60 | ND | ND | 0.02 | 0.25 |

| Brassica napus var. n. (turnip) | | | | | | | | |
| Mature root [3] | 0.01 ± 0.01 | 0.09 ± 0.01 | 0.17 ± 0.07 | 1.42 ± 0.03 | 0.01 ± 0.00 | ND | 0.03 ± 0.02 | 0.01 ± 0.00 |
| Mature leaf [3] | ND | 0.08 ± 0.01 | 0.15 ± 0.04 | 1.25 ± 0.21 | ND | 0.04 ± 0.01 | 0.11 ± 0.00 | 0.01 ± 0.00 |
| Seed [1] | ND | ND | 0.10 | 2.10 | ND | ND | 0.22 | 0.04 |

| Brassica napus (rapeseed) | | | | | | | | |
| Young root [1] | 0.04 | ND | 0.18 | 1.12 | ND | 0.02 | ND | 0.10 |
| Young leaf [1] | ND | ND | 0.25 | 2.10 | ND | 0.04 | ND | 0.45 |
| Flower bud [2] | 0.01 | ND | 0.45 | 1.35 | ND | 0.02 | ND | 0.08 |
| Flower [2] | ND | 0.02 | 0.45 | 0.96 | ND | 0.01 | ND | 0.04 |

Pec, 2-phenylethylamine [C₆H₅(CH₂)NH₂]; Dap, diaminopropane [NH₂(CH₂)₂NH₂]; Put, putrescine [NH₂(CH₂)₄NH₂]; Spd, spermidine [NH₂(CH₂)₃NH(CH₂)₄NH₂]; HSpd, homospermidine [NH₂(CH₂)₄NH(CH₂)₄NH₂]; TSpm, thermospermine [NH₂(CH₂)₃NH(CH₂)₃NH(CH₂)₄NH₂]; Spm, spermine [NH₂(CH₂)₃NH(CH₂)₄NH(CH₂)₃NH₂]; Agm, agmatine [NH₂C(NH)NH(CH₂)₄NH₂]. ND, not detected (< 0.005 μmol/g wet weight). [ ], number of samples. Values are shown as M (mean) [2 samples] or M (mean) ± SD (standard deviation) [3-4 samples].
μmol/g w.w.) and mature healthy roots (0.63 μmol/g w.w.). The agmatine level in the CB roots was 1/3 and 1/9 that in normal roots and non-infected mature healthy roots, respectively. The level of 2-phenylethylamine was lower in the CB roots than in the normal roots. A lower homospermidine concentration was detected in the CB roots than in the normal roots.

In the infected Komatuna, the concentration of 2-phenylethylamine was lower in the CB roots (0.02 μmol/g w.w.) than in the mature healthy roots (0.16 μmol/g w.w.). A two-fold higher level of spermidine was observed in the CB roots (2.22 μmol/g w.w.) than in the young roots and mature healthy roots, 1.02 and 1.19 μmol/g w.w., respectively. The spermine level was relatively high in the CB roots. The agmatine and homospermidine levels found in the young and mature healthy roots reduced by half in the CB roots.

Since a pure culture of a cercozoan P. brassicae belonging to the class Phytomxea of the phylum Cercozoa has not been reported, the polyamine profile of the protist is unknown. Putrescine and spermidine were found but tetra-amines were not detected in the heterotrophic protists, Cremosynas and Massisteria of the class Cercomonadiida and Thaumatomastix of the class Thaumatomastix belonging to the same phylum Cercozoa (Hamana, 2008). Although contamination of putrescine and spermidine from the protists was not excluded as a part of the clubroot polyamines, an increase in putrescine, spermidine and spermine levels and a decrease in agmatine level might have occurred in the CB roots in Brassica.

The putrescine, spermidine and spermine levels have been reported to be increased in turnip with clubroot (Walters and Shuttleton, 1985; Walters, 2003), but homospermidine and thermospermine levels have not been reported. In the present study, thermospermine constantly appeared at a similar level in the CB roots, normal roots and mature healthy roots in the three Brassica plants, indicating a difference in the defensive behavior of spermine and thermospermine against clubroot disease. In A. thaliana, spermine is not essential for normal growth but plays a role in salinity and thermal stress responses, and thermospermine has been shown to be involved in stem elongation (Kakehi et al., 2008; Naka et al., 2010; Sagor et al., 2013). Thermospermine as well as spermine suppress the multiplication of the cucumber mosaic virus in A. thaliana (Mitsuya et al., 2009; Sagor et al., 2012). Therefore, an increase in the spermidine level might serve as a major defense function against clubroot disease in Brassica root. It is clear that the relative levels of homospermidine against spermidine or total polyamines decreased during clubroot gall formation in the Brassica roots analyzed in the present study.

A decrease in the level of agmatine was detected in the CB roots of three Brassica plants while agmatine was accumulated in the A. thaliana roots in response to the infection with P. brassicae (Hanfrey et al., 2001). Although a high concentration of agmatine has been reported in leguminous root nodules caused by the infection of Bradyrhizobium (Fujihara, 2009), the present study showed that the 2-phenylethylamine level decreased in the Brassica plant CB roots, indicating that the production of the guanidinoamine and the aromatic amine is not stimulated in Brassica clubroot.

2. Polyamine profiles in other organs of Brassica plants

Polyamine concentrations in various organs of eight non-infected Brassica plants are shown in Table 1. 2-Phenylethylamine was found in some roots, leaves and flower buds of the Brassica crops, but cadaverine, a diamine produced by lysine decarboxylase from L-lysine, was not detected (not shown in Table 1). However, cadaverine appeared in the broccoli sprouts at the concentration of 0.10 μmol/g w.w. (not shown in Table 1), suggesting that lysine decarboxylase activity was induced selectively during the seedling phase in broccoli.

A low concentration of norspermine (0.02 μmol/g w.w.) was found in some Komatsuna tissues (not shown in Table 1). Supplement of authentic norspermine was reported to induce stem elongation in A. thaliana, in which endogenous norspermine was not found (Kakehi et al., 2010).

In cabbage, broccoli and Komatsuna, sprout roots and young roots showed a significant amount of homospermidine. The young leaves of cabbage and Komatsuna were rich in spermidine and spermine whereas mature leaves were rich in putrescine. The agmatine level in the mature leaves of broccoli (0.35 μmol/g w.w.) decreased in the old leaves (0.09 μmol/g w.w.) after the harvest of flower buds. In the mature leaves of the three Brassica plants, a difference in their putrescine levels between the infected and the healthy plants was observed, indicating a decrease in putrescine level of the leaves caused by clubroot disease.

In A. thaliana, it has been demonstrated that thermospermine is required for stem elongation (Kakehi et al., 2008) and that stems and flowers contain two- to three-fold more thermospermine compared with whole seedlings and mature leaves (Naka et al., 2010). Our results in broccoli that the thermospermine level in the sprout stems was higher than in the sprout root supported their findings.

High agmatine concentrations (0.35 – 2.82 μmol/g w.w.) were observed in the flower buds of broccoli, Sticksenor, cauliflower and Romanesco as well as in broccoli sprouts (0.98 – 3.26 μmol/g w.w.). The accumulated agmatine in the flower buds seems to lead to subsequent polyamine synthesis for flower and seed formation in the four Brassica plants. High spermidine and spermine levels and a low
thermospermine level were found in the seeds of eight *Brassica* plants and could be beneficial against thermal and dry stresses, as proven in leguminous and gramineous seeds (Hamana et al., 1992, 1996; Hamana and Matsuzaki, 1993). In contrast, differences in the putrescine and agmatine levels were observed in the seeds among the eight *Brassica* plants.

Polyamines have been considered to play a role in the longevity in living organisms and ingestion of polyamines in food has been proposed to combat the decrease in mammalian cellular polyamine levels caused by aging (Nishimura et al., 2006; Soda et al., 2009, 2013; Matsumoto et al., 2011). The seeds of 46 leguminous crop plants contained putrescine, spermidine, spermine and agmatine at the concentrations of 0.10 – 1.11, 0.70 – 1.56, 0.03 –1.20 and 0.00 – 0.73 \( \mu \text{mol/g w.w.} \) respectively (Hamana et al., 1992, 1996; Hamana and Matsuzaki, 1993). The seeds of three gramineous crop plants contained putrescine, spermidine, spermine and agmatine at the concentrations of 0.30 – 0.95, 0.25 – 1.51, 0.04 – 0.84 and 0.00 – 0.15 \( \mu \text{mol/g w.w.} \) respectively (Hamana et al., 1994). In comparison with leguminous and gramineous seeds, the leaves of cabbage and Komatsuna were rich in putrescine, spermidine and spermine, the flower buds of broccoli and cauliflower were rich in agmatine and spermine, and broccoli sprouts were rich in putrescine, spermidine and agmatine as shown in the present study. Therefore, the *Brassica* organs are useful as nutrient-rich vegetables for polyamine intake.

**Acknowledgements**

We thank Mr. Shigetake Takahashi and Mr. Hiroyuki Fuji (Kuroiwa Survey Design Office Co.) for the cultivation of *Brassica* crop plants.

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