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POLYAMINE ANALYSIS OF CROWN GALL AND NORMAL ROOT, STEM, LEAF, FRUIT AND SEED IN *ROSEALES* AND *FAGALES* PLANTS

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ABSTRACT

To determine taxonomically specific plant polyamines and organ-specific plant polyamines, the polyamines acid-extracted from crown galls in addition to normal roots, stems, leaves, fruits, and seeds of 11 *Roseales* plants and a *Fagales* plant of the phylum Angiospermae were quantitatively analyzed by high-performance liquid chromatography and high-performance gas chromatography. Putrescine, spermidine and spermine were detected ubiquitously as the major polyamines present in the plant organs. The crown galls on the stem of cherry (*Cerasus*), apple (*Malus*) and oak (*Quercus*), caused by the infection of phytopathogenic *Rhizobium* (*Agrobacterium*), were rich in homospermidine in addition to the three major polyamines and minor norspermine and/or thermospermine also found in the normal stems. The concentrations of the three polyamines in the young leaves were decreased in the fallen (aged) leaves in two mulberry (*Morus*) samples. High homospermidine level was observed in the roots of the cherry and mulberries. In addition to similar distribution of the three major polyamines, diaminopropane, cadaverine, norspermidine and homospermidine were detected sporadically as a minor polyamine in the eight *Roseales* fruits, apple, loquat (*Eriobotrya*), fig (*Ficus*), mulberry, apricot (*Prunus*), peach (*Prunus*), plum (*Prunus*) and pear (*Pyrus*). In pear fruit, the skin of the fruit had a lower polyamine level. Cellular polyamines function in adapting to the environment of plant growth.

Keywords: Crown gall, *Fagales* plant, fruit, polyamine, *Roseales* plant

INTRODUCTION

Cellular polyamines and its derivatives found in plants are involved in plant cell proliferation and differentiation and play a defensive role against various stresses and infectious diseases, and affect symbiotic and parasitic processes by microbes in higher land plants (Kusano *et al.*, 2008; Minocha *et al.*, 2014; Naka *et al.*, 2010; Takahashi and Kakehi, 2010). It has been suggested that high-basic and low-molecular polyamines interact with the acidic components ATP, RNA and DNA in plant cells and function in adapting to the environment of plant growth. We have analyzed approximately 35 kinds of biogenic polyamines of the polyamine fraction acid-extracted from plant organs by high-performance liquid chromatography (HPLC) and high-performance gas chromatography (HPGC) developed in our laboratory to specialize in polyamine analysis, and comprehensively examined endogenous polyamines in root, leaf, pod, flower and seed of vegetables belonging to the orders *Brassica* and *Solanales* (Hamana *et al.*, 2015, 2017; Niitsu *et al.*, 2014), in osmotic-resistant body of aquatic plants (Hamana *et al.*, 1994, 1998, 2000), and in seed and seedling (or sprout) of agricultural crop plants belonging to the families *Fabaceae* (formerly *Leguminosae*) (Hamana *et al.*, 1996, 2019) and *Poaceae* (formerly *Gramineae*) (Hamana *et al.*, 1994).

On the other hand, we analyzed polyamines in *Brassica* clubroots caused by the infection of a cercozoan, *Plasmodiophora brassicae*, in root-knot galls of tomato and eggplant by a root-knot nematode, *Meloidogyne incognita*, and in powdery scab, a disease of potato tuber by a cercozoan, *Spongospora subterranean* (Hamana *et al.*, 2015, 2017). Polyamine analyses of the coralloid root of a fern palm, *Cycas revolute* (the phylum *Gymnospermae*), containing a symbiotic cyanobacterium, *Anabaena cycadea*, and the closed leaf pocket of the ferns *Azolla imbricate* and *A. japonica* (the phylum *Pteridophyta*) containing *Anabaena azollae*, had been reported, respectively (Hamana and Mastuzaki, 1985; Hamana *et al.*, 1988). Legumes belonging to the family *Fabaceae* are notable in that most of them have symbiotic nitrogen-fixing rhizobia *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* (Fujihara, 2009), therefore we attempted a polyamine analysis of leguminous root nodules in comparison with normal roots (Hamana *et al.*, 2019). Phytopathogenic tumor inducing (Ti) strains of *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*), *R. rhizogenes*, *R. vitis*, *R. rubi* and *R. larrymoorei* are the causal agent of crown gall disease in higher land plants, however, another pathogenic hairy root inducing (Ri) and nonpathogenic strains are included within the genus (Young *et al.*, 2001). Though we have shown cellular polyamine distribution profiles of rhizobia and phytopathogenic *Agrobacterium* (*Rhizobium*) species (Hamana *et al.*, 1988; 1989, 1990, 1993), polyamine analysis of crown galls was essential for the polyamine research of agricultural plants.

MATERIALS AND METHODS

Plant organs and crown galls

Plant organs, including crown galls on stems, branches or limbs (not distinguished among three organs in the present report) of cherry (*Cerasus sargentii*), apple (*Malus pumice*) and oak (*Quercus serrate*) were collected by permission in Tsumagoi Garden, Numata Fruit Farm and Maebashi Garden at Gunma Prefecture, Japan. Roots, leaves, false fruits (fruits in the present report) and seeds of the plants such as loquat, fig tree, apricot, peach, plum and pear were collected by permission at the Momonoki river side, Maebashi. Organs of natural crabapple and cultivated mulberry were collected in a forest and a field, respectively in Tsumagoi. Whole fruit of fig tree and mulberry whereas fruit parts excluded seeds, and skins (peels) in other six *Roseales* plants were used for a rough fruit-polyamine extraction.

Polyamine analysis

Organs (10-100g) were homogenized in the same weight of 10% (1.0M) perchloric acid (PCA) by a mixer. After extraction with 5% PCA three times, the supernatant of the PCA extract was applied to a column (3cm I.D. x 1cm) of a cation-exchange resin, DOWEX W50x8 (Dow Chemical Co., Michigan, USA), and then polyamines were concentrated by the elution 6M HCl from the column. HPLC of the polyamine fraction were performed on a Hitachi L6000 (Tokyo, Japan) using a column of cation-exchange resin, Hitachi 2619F (=Hitachi 2720) (4mm I.D. x 50mm) (Hamana *et al.*, 2015). HPGC on a SHIMADZU GC-17A (Kyoto, Japan) and HPGC-mass spectrometry (MS) on a JEOL JMS-700 (Tokyo, Japan), equipped with a long capillary column (0.32mm I.D. x 30m) of Inert Cap 1MS (GL Sciences, Tokyo, Japan) were performed after heptafluorobutyrylation of the polyamine fraction (Niitsu *et al.*, 2014; Hamana *et al.*, 2015, 2019). Molar concentrations of endogenous polyamines per gram of wet weight of organ ($\mu\text{mol/g}$ wet weigh) estimated from the charts of the HPLC, HPGC and HPGC-MS analyses of the polyamine extracts are shown in Table 1.

RESULTS AND DISCUSSION

Though cherries have been cultivated for cherry blossom and cherry fruit, crown galls are found frequently on its stem. Crown galls are also frequently found on the stem of apple trees in apple fruit farms and oak trees used for Shiitake mushroom cultivation. In the crown galls on the stem of cherry (*Cerasus sargentii*) and apple (*Malus pumila*) belonging to the order *Roseales* and oak (*Quercus serrata*) of the order *Fagales* were similarly rich in homospermidine (HSpd) in addition to major spermidine (Spd) and spermine (Spm) and minor norspermine (NSpm) and thermospermine (TSpd) (Table 1). Higher levels of HSpd, NSpd and TSpd were found in the

crown galls rather than normal stems in the cherry and apple. In the oak, HSpd and agmatine (Agm) increased in the old crown galls. Lower level of Spm was found in the old crown galls of the three plants.

Table 1: Polyamine concentrations in *Roseales* and *Fagales* plant organs and crown galls

Plant species	organs	Polyamines (μmol/g wet weight)												
Eucots in Angiosperms (Phylum Angiospermae)		Dap	Put	Cad	NSpd	Spd	HSpd	NSpm	TSpm	Spm	AHSpd	Can	HSpm	Agm
Order <i>Roseales</i>		3	4	5	33	34	44	333	334	343	344	434	444	
<i>Cerasus sargentii</i> (<i>Prunus sargentii</i>) (Cherry)	root	-	0.02	-	-	0.56	0.20	-	0.02	0.14	-	-	-	-
	"Ouyama-sakitem (young)	-	0.03	-	-	1.20	0.15	0.01	-	0.10	-	-	-	-
	(old)	-	0.02	-	-	0.58	0.02	0.01	0.01	0.27	-	-	-	-
	crown gall (young)	0.10	0.22	-	-	1.10	0.26	0.04	-	0.20	-	-	-	-
	(young)	-	0.05	-	-	0.70	0.20	0.02	0.03	0.32	-	-	-	-
	(old)	-	0.40	-	-	1.17	0.99	-	-	0.08	-	-	-	-
<i>Malus pumila</i> (Apple)	"Fuji-ring stem	0.10	0.10	-	-	0.72	0.10	-	-	0.03	-	-	-	-
	crown gall (old)	0.03	0.15	-	-	0.75	0.33	0.02	-	0.02	-	-	-	-
<i>Malus toringo</i> (<i>Malus sieboldii</i>) (Toringo crabapple)	"Zumi leaf	0.02	0.40	0.02	-	1.80	0.20	-	-	0.40	-	-	-	0.04
	fruit	-	0.20	-	-	1.40	0.02	-	-	0.41	-	-	-	0.30
<i>Eriobotrya japonica</i> (Japanese loquat)	"Biwa" fruit	-	0.44	0.07	-	2.10	0.10	-	-	0.15	-	-	-	-
	seed	-	0.17	0.02	-	1.60	0.02	-	-	0.27	-	-	-	-
	leaf	-	0.02	0.15	-	1.30	-	-	-	0.19	-	-	-	-
<i>Ficus carica</i> (Fig tree)	"Ichijiku leaf	-	0.10	0.02	-	0.53	-	-	-	0.03	-	-	-	-
	fruit	-	1.30	-	-	0.60	-	-	-	0.37	-	-	-	0.04
<i>Morus alba</i> (Mulberry)	"Maguw" root	-	0.03	-	-	1.36	0.45	-	-	0.15	-	-	-	0.10
	leaf (young)	-	0.52	-	-	1.23	-	-	-	0.52	-	-	-	0.05
	(old)	-	0.05	-	-	0.63	0.01	-	-	0.71	-	-	-	-
	fallen leaf	-	0.10	-	-	0.88	-	-	-	0.15	-	-	-	-
	fruit	-	0.30	-	-	1.50	-	-	-	0.20	-	-	-	-
<i>Morus bombycis</i> (Mulberry)	"Yamaguw" root	-	0.25	-	-	0.65	0.25	-	-	0.03	-	-	-	0.02
	leaf (young)	-	0.30	-	-	1.80	-	-	-	0.30	-	-	-	-
	fallen leaf	-	0.04	-	-	0.55	-	-	-	0.03	-	-	-	-
	fruit	-	0.80	0.10	-	0.25	-	-	-	0.10	-	-	-	-
<i>Prunus mume</i> (Japanese apricot)	"Ume fruit	-	0.90	0.01	-	0.25	-	-	-	0.01	-	-	-	0.03
<i>Prunus persica</i> (Peach)	"Mom fruit	0.03	0.85	-	-	1.40	0.01	-	-	0.15	-	-	-	-
<i>Prunus salicina</i> (Plum)	"Sumomo-soldum fruit	-	1.80	0.01	-	1.90	0.02	-	-	0.10	-	-	-	0.02
<i>Pyrus pyrifolia</i> (Nashi pear)	"Nasl fruit	-	0.96	-	0.02	1.40	0.01	-	-	-	-	-	-	0.03
	fruit skin	-	0.85	-	-	1.04	-	-	-	-	-	-	-	1.00
Order <i>Fagales</i>														
<i>Quercus serrata</i> (Pin oak)	"Konar crown gall (young)	-	0.06	-	-	0.74	0.05	-	0.01	0.82	-	-	-	-
	(young)	-	0.03	-	-	0.60	0.05	-	-	0.80	-	-	-	-
	(old)	-	0.10	-	-	0.80	0.35	-	-	0.46	-	-	-	0.33
	(old)	0.10	0.10	0.02	-	0.80	0.10	-	0.01	0.65	-	-	-	0.04

Alphabetical abbreviations and numeric codes (number of methylene (CH₂) groups between amino (NH₂) or imino (NH) groups) of polyamines are used. Dap, diaminopropane [NH₂(CH₂)₃NH₂]; Put, putrescine [NH₂(CH₂)₄NH₂]; Cad, cadaverine [NH₂(CH₂)₅NH₂]; NSpd, norspermidine [NH₂(CH₂)₃NH(CH₂)₃NH₂]; Spd, spermidine [NH₂(CH₂)₃NH(CH₂)₄NH₂]; HSpd, homospermidine [NH₂(CH₂)₄NH(CH₂)₄NH₂]; NSpm, norspermine [NH₂(CH₂)₃NH(CH₂)₃NH(CH₂)₃NH₂]; TSpm, thermospermine [NH₂(CH₂)₃NH(CH₂)₃NH(CH₂)₄NH₂]; Spm, spermine [NH₂(CH₂)₃NH(CH₂)₄NH(CH₂)₃NH₂]; AHSpd, aminopropylhomospermidine [NH₂(CH₂)₃NH(CH₂)₄NH(CH₂)₄NH₂]; Can, canavamine [NH₂(CH₂)₄NH(CH₂)₃NH(CH₂)₄NH₂]; HSpm, homospermine [NH₂(CH₂)₄NH(CH₂)₄NH(CH₂)₄NH₂]; Agm, agmatine [NH₂C(NH)NH(CH₂)₄NH₂]. -, not detected (<0.005 μmol/g wet weight). (), popular name. " ", species or variety name in Japanese.

In phytopathogenic *Rhizobium* (*Agrobacterium*) species, HSpd is the major polyamine and NSpd, Spd, NSpm, Spm and TSpm are almost absent (Hamana *et al.*, 1990, 1993). It is suggested that high HSpd level in the crown galls was derived from *Agrobacterium* polyamines grown in the stem. In the present report, NSpm and TSpm slightly increased and Spm decreased in the crown galls after the *Agrobacterium* infection. Cadaverine (Cad), aminobutylcadaverine (ABCad) and methylated polyamines found in some leguminous root nodules including nitrogen-fixing rhizobia (absent in Cad and ABCad) (Hamana *et al.*, 2019) were not found in the crown galls of the *Roseales* and *Fagales* plants.

Mulberries (*Morus alba* and *Morus bombycis*) belongs to *Roseales* and its leaf and fruit are available as a food as well as essential for silkworm culture. Putrescine (Put), Spd, and Spm are the major polyamines of the leaves and fruits of the two mulberries (Table 1). The concentrations of the three polyamines in the young leaves were decreased in old and fallen yellow leaves. This is the first report on the comparison of polyamine distributions between photosynthetically active young leaves and non-photosynthetic fallen leaves (aged leaves), a typical decrease of polyamine level with aging in plant cells. HSpd was detected in the roots as a major polyamine.

In similar polyamine distributions and concentrations of the eight *Roseales* fruits of 8 species analyzed; the apple fruit is rich in Spm and Agm, the loquat fruit contained homospermidine as a major polyamine, the fig is rich in Put, the fruits of two mulberries contained Put, Spd and Spm as the major polyamines, the apricot fruit had a low level of Spm, the peach fruit contained Dap and HSpd as the minor polyamines, the plum fruit contained Put, Cad, HSpd as the minor polyamines, and pear fruit contained NSpd as a minor polyamine and lacked in Spm, as shown in Table 1. The fruits of the eight species showed a variety of minor polyamine distributions. Polyamine analysis of popular *Roseales* fruits for food usage contribute as some nutritional information on polyamine ingestion from agricultural products with divers functions in human health and disease (Handa *et al.*, 2018).

The loquat seed was relatively rich in the tetra-amine Spm. It has been shown that leguminous seeds are rich in tetra-amines and penta-amines (Hamana *et al.*, 1996, 2019) and gramineous seeds are poor in tetra-amines (Hamana *et al.*, 1994).

It is speculated that skin of fruit performs as a protector from light and heat in atmosphere, on the other hand, skin of the root encounters microbial infection in the ground. The fruit skin of pear had a lower polyamine level, however, agmatine was detected as a major polyamine (Table 1). In our previous report, skins of tomato and eggplant fruits showed lower polyamine levels, whereas skins of potato tubers and sweet potato tuberous roots showed higher polyamine levels (Hamana *et al.*, 2017), suggesting different functions of polyamines in adapting to the environment of plant growth.

Tetra-amines, aminopropyl homospermidine (AHSpd), canavamine (Can) and homospermine (HSPm) found in *Fabaceae* (Hamana *et al.*, 1996, 2019), 2-hydroxypolyamines found in *Poaceae* (Hamana *et al.*, 1994) and methylpolyamines found in *Fabaceae* (Hamana *et al.*, 1996, 2019) have not been found in *Roseales* and *Fagale* plants. In addition to phylogenetic polyamine distribution catalogue in various taxa of agricultural plant products and polyamine distribution profile in differentiated plant organs, the present plant polyamine analyses are useful to know change of cellular polyamine profiles during the infection and symbiosis of microbes in plants (Tiburcio and

Alcazar, 2018).

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