

Levels of Polyamines and Kinetic Characterization of Their Uptake in the Soybean Pathogen *Phytophthora sojae*

M. Constantine Chibucos and Paul F. Morris*

Department of Biological Sciences, Bowling Green State University, Bowling Green, Ohio 43403

Received 5 August 2005/Accepted 2 March 2006

Polyamines are ubiquitous biologically active aliphatic cations that are at least transiently available in the soil from decaying organic matter. Our objectives in this study were to characterize polyamine uptake kinetics in *Phytophthora sojae* zoospores and to quantify endogenous polyamines in hyphae, zoospores, and soybean roots. Zoospores contained 10 times more free putrescine than spermidine, while hyphae contained only 4 times as much free putrescine as spermidine. Zoospores contained no conjugated putrescine, but conjugated spermidine was present. Hyphae contained both conjugated putrescine and spermidine at levels comparable to the hyphal free putrescine and spermidine levels. In soybean roots, cadaverine was the most abundant polyamine, but only putrescine efflux was detected. The selective efflux of putrescine suggests that the regulation of polyamine availability is part of the overall plant strategy to influence microbial growth in the rhizosphere. In zoospores, uptake experiments with [1,4-¹⁴C]putrescine and [1,4-¹⁴C]spermidine confirmed the existence of high-affinity polyamine transport for both polyamines. Putrescine uptake was reduced by high levels of exogenous spermidine, but spermidine uptake was not reduced by exogenous putrescine. These observations suggest that *P. sojae* zoospores express at least two high-affinity polyamine transporters, one that is spermidine specific and a second that is putrescine specific or putrescine preferential. Disruption of polyamine uptake or metabolism has major effects on a wide range of cellular activities in other organisms and has been proposed as a potential control strategy for *Phytophthora*. Inhibition of polyamine uptake may be a means of reducing the fitness of the zoospore along with subsequent developmental stages that precede infection.

The genus *Phytophthora* consists of some 60 species of diploid, alga-like, plant-pathogenic oomycetes (17) classified in the kingdom Stramenopiles, which are now grouped in the Chromalveolata (48). Collectively, members of the genus *Phytophthora* infect more than 1,000 plant species worldwide (17), causing damage to both natural plant populations and economically important crops, including tomato, potato, soybean, and cocoa. *Phytophthora sojae* attacks soybeans (*Glycine max*) at all growth stages, resulting in significant decreases in yield in spite of the deployment of race-specific resistance in soybean cultivars (46).

Under flooding conditions, vegetative hyphae of *P. sojae* in the soil produce vertical branches with terminal sporangia. Each sporangium releases 20 to 30 single-cell asexual zoospores that act as the primary dispersal and infection agents for the pathogen (17). Zoospores contain both lipid and mycolaminarin reserves which provide the main source of energy for zoospore motility (11). Exogenous compounds are thought to serve as signals that promote chemotaxis and encystment rather than as nutrient sources (15). Both the zoospores and the hyphae are attracted by isoflavones released from soybean roots (38, 39). When a zoospore contacts a host root, it loses its flagella, secretes an adhesive mucilaginous glycoprotein, and forms a cell wall. A hyphal germ tube emerges from the germinating cyst, penetrates between host cells, and produces a

hyphal network that extracts nutrients from the host. When the nutrients are exhausted, the hyphae form sexual gametangia. Fertilized oospores have thick cell walls that enable the pathogen to remain dormant in the soil over winter (17).

Polyamines are ubiquitous aliphatic cations present in all life forms that can affect diverse cellular processes, such as nucleic acid synthesis, protein synthesis, membrane permeability, and development (16). In plants, the formation of hydroxycinnamic acid conjugates of polyamines and their subsequent deposition into plant cell walls are thought to be an integral part of plant defense against pathogen ingress (18). Polyamines are equally important for the invading pathogen, and perturbation of polyamine metabolism is a potential means of control (9, 44). In *Phytophthora infestans* application of the putrescine biosynthesis inhibitor α -difluoromethylornithine reduced hyphal growth, but this reduction could be reversed by adding exogenous polyamines (9). Significant quantities of polyamines may occur in the soil (54), plant phloem (2, 13, 21), and root nodules (22, 23, 40). Polyamine uptake has been studied in many microorganisms, including bacteria (27, 34), yeasts (4, 28), protozoans (26, 35), and soil fungi (20, 37). Polyamine uptake can be used to maintain cellular homeostasis, and uptake of polyamines by swimming *P. sojae* zoospores, heretofore believed to be dependent on internal stores until host contact (8, 11, 12), could increase zoospore survival and aid the development of the germinating cysts.

The objectives of this research were (i) to determine basal levels of free and conjugated polyamines in the motile zoospores and vegetative hyphae of *P. sojae* and young soybean root tissues; (ii) to characterize the kinetics of polyamine up-

* Corresponding author. Mailing address: Department of Biological Sciences, Bowling Green State University, Bowling Green, OH 43403. Phone: (419) 372-0481. Fax: (419) 372-2024. E-mail: pmorris@bgsu.edu.

take in swimming zoospores; and (iii) to determine whether polyamines are released into the rhizosphere by soybean roots. We hypothesized that different life stages of *P. sojae*, as well as soybeans, contain different levels of polyamines that coincide with the multiple effects that these compounds have on cellular processes in other organisms. We also hypothesized that zoospores exhibit high-affinity putrescine and spermidine uptake consistent with Michaelis-Menten saturation kinetics. Finally, since plant roots secrete a wide range of compounds into the rhizosphere, we hypothesized that polyamines also are excreted. Knowledge of pathogen polyamine biology and rhizosphere polyamine dynamics may provide new insights into mechanisms for pathogen control.

MATERIALS AND METHODS

Organisms. *P. sojae* (University of California-Riverside *Phytophthora* Collection strain p6497) was maintained on V8 agar at 17°C and was subcultured weekly (17). Zoospores were produced from hyphae by repeated plate washing as described previously (17). Typical average concentrations were 1.5×10^5 to 4.5×10^5 zoospores/ml. Hyphal mats were grown with shaking (50 rpm) in 250-ml flasks containing 100 ml liquid CV8 medium (17) or on yeast carbon agar (YCA) plates (Remel, Lenexa, KS) at room temperature (23 to 25°C). Each flask was inoculated with a 0.5-cm plug of 1-week-old hyphae from a V8 plate. Soybean seeds were surface sterilized and then grown for 5 days in vermiculite at 20°C with 16 h of daylight.

Polyamine stock solutions and radiolabeled polyamines. Polyamine stock solutions (1 mM) were prepared in water or 0.1 N HCl and stored at -20°C. These stock solutions were further diluted in water or 0.01 N HCl for use in experiments. [1,4-¹⁴C]putrescine (3.96 Gbq/mmol) and [1,4-¹⁴C]spermidine (4.14 Gbq/mmol) were obtained from Amersham Biosciences (Piscataway, NJ). Radiolabeled polyamines were diluted 1:100 in water and stored at 4°C until they were used.

Zoospore chemotaxis and swimming longevity. The tropic response of zoospores to putrescine was assessed with a capillary assay (39). The effect of exogenous polyamines on swimming longevity was assessed by incubating cells at 17°C in solutions containing various concentrations (10 μM to 10 mM) of different polyamines and counting the number of swimming cells.

Hyphal polyamine levels and growth with polyamine as the sole nitrogen source. After growing in liquid CV8 medium for 14 days, hyphal mats were placed on a 1-mm-mesh strainer, and the original agar plug was removed with tweezers. The hyphae were rinsed with 500 ml of 1 mM NaCl and 500 ml of distilled water and then vacuum dried with a 0.45-μm Nalgene vacuum filtration apparatus (Nalge Nunc International, Naperville, IL) and weighed. Three milliliters of 10% (vol/vol) perchloric acid was added to each 300 mg of tissue, and samples were frozen at -80°C.

Before we determined if hyphae could utilize polyamines as a sole nitrogen source, hyphae were depleted of excess N by subculturing 0.5-cm-diameter plugs of actively growing hyphae from V8 agar to glucose-containing YCA plates and grown for 14 days. Hyphal plugs from the leading edge of the YCA plates were transferred to plates containing V8 agar, YCA, or YCA with 110 μM putrescine, 110 μM cadaverine, 110 μM spermidine, or 110 μM putrescine and 110 μM spermidine.

Soybean seedling polyamine titer and polyamine exudation. Plants were removed from vermiculite, and the roots were rinsed with 10 ml water, which was saved for polyamine analysis. Roots were placed in 15-ml centrifuge tubes with 10 ml water for 1 h and then transferred to new tubes with fresh water and soaked for an additional 1 h. One milliliter of 10% (vol/vol) perchloric acid was added to each tube for polyamine extraction. Roots were macerated with a razor blade, combined with 3 ml of 10% (vol/vol) perchloric acid, and stored at -80°C until homogenization and complete acid extraction.

Zoospore and V8 agar plate wash polyamine collection. One-milliliter aliquots of plate wash liquid from soaked and washed V8 agar plates containing hyphae (2 ml recovered per plate) were transferred to 50-ml tubes, mixed with 1 ml of 10% (vol/vol) perchloric acid, and frozen at -80°C prior to polyamine analysis. Three milliliters of 6-h-old zoospores or 6 ml of 36-h-old zoospores was condensed by combining liquid from plates in 50-ml tubes, followed by centrifugation (7,600 × g, 10 min, 15°C). The supernatant was decanted and replaced with 15 ml of 1 μM NaCl. After centrifugation, the supernatant was decanted, and 3

ml of 10% perchloric acid was added to each pellet. The tubes were vortexed vigorously and frozen at -80°C until acid hydrolysis and derivatization.

Acid extraction of polyamines from media, zoospores, hyphae, and soybean tissues. Polyamines were extracted from all acidified samples (19). Extraction with perchloric acid released soluble, nonconjugated ("free") polyamines. Acid-heat hydrolysis of the perchloric acid-soluble fractions in 6 N HCl yielded soluble, conjugated polyamines. Acid-heat hydrolysis of perchloric acid-insoluble pelleted cellular material yielded insoluble, conjugated ("bound") polyamines (49). Acidified media, plate wash liquids, and soybean root exudates did not require processing beyond the initial addition of perchloric acid to release free polyamines. Hyphae, zoospores, and root tissues required maceration and acid-heat hydrolysis. Zoospores in 3 ml perchloric acid were thawed and vortexed vigorously to complete the extraction of free polyamines. Two milliliters of the zoospore suspension was transferred to a centrifuge tube for benzylation, and 1 ml was transferred to an amber borosilicate glass ampoule for hydrolysis. After addition of 1.0 ml of 12 N HCl and 25 nmol of 1,7-diaminoheptane (internal standard), the ampoules were flame sealed by tip pulling and incubated at 110°C for 16 h to release both soluble and insoluble conjugated polyamines.

Soybean tissue and hyphae in 3 ml perchloric acid were thawed and macerated with a handheld glass tissue homogenizer, after which the slurry was transferred to a centrifuge tube and incubated for 4 h on ice. Extracts were centrifuged (13,000 × g, 60 min, 4°C). The supernatant was aspirated and saved, and the pellets were resuspended in 600 μl of 10% (vol/vol) perchloric acid. Resuspension, centrifugation, and aspiration of pellets were repeated up to five times to extract the soluble polyamines from a pellet. All but 1 ml of the supernatant was transferred to a 50-ml centrifuge tube for polyamine derivatization. Either 1 ml of the supernatant or the entire pellet resuspended in 1 ml perchloric acid was transferred to an amber borosilicate glass ampoule. One milliliter of 12 N HCl and 25 nmol of 1,7-diaminoheptane were added to ampoules before sealing by tip pulling and acid hydrolysis for 16 h at 110°C. Hydrolysates were transferred to 50-ml tubes for benzylation.

Derivatization of polyamines with benzoyl chloride. Acid-extracted biogenic polyamines and acidified polyamine standards were derivatized with benzoyl chloride by using a modified version of the Schotten-Baumann technique (19, 43). All reactions were performed in 50-ml polypropylene screw-cap centrifuge tubes in a chemical hood. Either 2 μM of 1,6-diaminohexane or 25 nmol of 1,7-diaminoheptane was added as an internal standard to each biogenic polyamine sample. To each 50-ml tube containing 1 to 2 ml of acidified polyamines, 2 to 3 ml of 10 N NaOH was added, followed by 20 μl of benzoyl chloride. After vortexing for 10 s, the tubes were incubated for 30 min at room temperature (20 to 25°C) with periodic vortexing. Two milliliters of saturated NaCl was added, and the solutions were mixed by vortexing for 10 s. Benzoyl-polyamine derivatives were extracted by adding 2 ml diethyl ether, followed by gentle end-over-end mixing. After separation, 1 ml of the organic phase was transferred to a 2-ml microcentrifuge tube and evaporated to dryness under a stream of air. Dry benzoyl-polyamines were resuspended in 60 μl of high-performance liquid chromatography (HPLC)-grade methanol, followed by addition of 60 μl water. Resuspended benzoyl-polyamines were analyzed by HPLC.

HPLC and polyamine standard curves. Reverse-phase HPLC was performed with a Hewlett-Packard 1050 series HPLC with a quaternary pump and a diode array detector (200 to 600 nm). Benzoyl-polyamines were separated on a Microsorb-MV 5 m/100 Å C₁₈ column (Rainin Instrument Company, Woburn, MA). Buffer A was 25 mM sodium phosphate (pH 6.0)-10% methanol, and buffer B was 100% methanol.

The initial conditions were 46% buffer A and 54% buffer B at a flow rate of 1 ml/min. From 0 to 3 min, the buffer B concentration was increased at a rate of 0.33%/min, from 3 to 5 min the buffer B concentration was increased at a rate of 5%/min, and from 5 to 7 min the buffer B concentration was increased at a rate of 17.5%/min. At 7 min the flow rate was changed to 1.25 ml/min and ramped to 1.5 ml/min by 8 min. This program separated 1,2-diaminopropane, putrescine, and cadaverine, which contain three, four, and five carbons, respectively. The diode array detector was set to record A_{200} to A_{400} . For polyamine analyses either A_{240} or A_{250} was used.

Benzoylated polyamine standards also were separated by HPLC and used to generate standard regression curves of chromatographic absorbance versus moles of standard compound. Samples were regressed against standards to determine sample loss (internal standards) and polyamine quantity.

Gas chromatography-mass spectrometry. HPLC peaks were collected for confirmation of identity by gas chromatography-mass spectrometry for putrescine and spermidine from zoospores and hyphae and cadaverine from roots. HPLC column eluates were collected from the waste line after they passed through the UV detector and were purified with a Prevail C₁₈ Maxi-Clean cartridge (Alltech, Deerfield, IL). Once they were dry, cleaned samples were suspended in 30 μl of

either dichloromethane (for putrescine) or ether (cadaverine or spermidine), and then 1 μ l of each suspension was injected into a Shimadzu (Kyoto, Japan) GC-17A/GCMS-QP5050A gas chromatograph-mass spectrometer. The gas chromatograph injector temperature was set at 325°C, and the column was a ZB-5 column (Phenomenex, Torrance, CA). Following injection, the column was maintained at 70°C for 2 min, and the temperature was then raised at a rate of 23°C/min until it reached 300°C.

Radiolabeled polyamine uptake protocol. One milliliter of zoospores (1.5×10^5 to 4.5×10^5 zoospores/ml) was transferred to a 2.0-ml microcentrifuge tube by using a wide-bore pipette, and this was followed by addition of a 100- μ l aliquot of an appropriately diluted unlabeled polyamine solution. A 125- μ l aliquot of 40% glycerol was layered into the bottom of the tube, and a 125- μ l layer of 60% glycerol was added below the 40% glycerol layer. Radiolabeled polyamine (10^5 dpm, 50 μ l) was added to the zoospore layer with gentle mixing. The experiments were performed for 5 min (except for the time-dependent uptake experiment). The uptake of polyamine was halted by centrifugation ($7,000 \times g$, 3 min, 25°C).

The radioactive supernatant and upper 40% glycerol layers were removed, and a 1-ml aliquot of 50% methanol was used to wash the inside of the tube down to the 60% glycerol layer. The supernatant and the 60% glycerol layer were removed, and a second 1-ml aliquot of 50% methanol was used to extract the glycerol layer surrounding the pellet. The pellet was resuspended in 50 μ l of Scintiverse E (Fisher Scientific, Pittsburgh, PA) and transferred to a glass scintillation vial containing 10 ml Scintiverse E. The incorporated radioactivity was quantified by liquid scintillation counting (LS-7000; Beckman Coulter, Fullerton, CA).

Correction for NSB. To correct for nonspecific binding (NSB), experimental replicates were centrifuged ($2,500 \times g$, 1 min) and washed immediately after addition of radiolabeled polyamines. In kinetic experiments in which the uptake of one substrate at several concentrations was measured, NSB replicates were prepared for a range of concentrations and a linear regression curve was generated since NSB increased linearly with concentration. In kinetic experiments with inhibitors, NSB was determined for each replicate since NSB also was affected by the inhibitor used.

Linearity of label uptake. Zoospores were incubated in the presence of radiolabeled putrescine or spermidine for 0 to 12 min to determine the optimal time interval for uptake experiments. Zoospores also were preincubated in unlabeled putrescine (3 to 30 μ M) for 30 min prior to uptake of labeled putrescine in order to confirm that uptake was consistent and linear at the concentrations tested.

Saturation kinetics experiments. The concentrations of spermidine and putrescine suitable for uptake studies ranged from medium levels (1 to 5 μ M) to 30 μ M, based on pilot experiments. Two to five replicates were used for each concentration. For all experiments, exogenous polyamines in the media were quantified by HPLC, and the values were used to determine total polyamine uptake. A Michaelis-Menten kinetics curve was generated by subtracting concentration-dependent NSB from total uptake.

Blocking putrescine/spermidine transporters. The uptake of radiolabeled putrescine or spermidine was measured in the presence of 15 μ M or 100 μ M unlabeled putrescine and spermidine, either singly or in combination. Three replicates were performed. The results were expressed as percentages of the total label taken up by the cells and were analyzed with a nonparametric Kruskal-Wallis test (SPSS, Chicago, IL). In a separate experiment 10^5 dpm of [1,4- 14 C]putrescine was added to zoospores that had been incubated in the presence of 25 μ M cadaverine or a no-polyamine control. This experiment was done twice.

RESULTS

Tropic response and swimming duration in zoospores. *P. sojae* zoospores did not exhibit a chemotactic response (either positive or negative) to any of the polyamines used in this study. The presence of polyamines at a biologically relevant range of concentrations in the medium had no effect on the swimming longevity of the zoospores. Concentrations of any of the polyamines tested that were more than 100 μ M reduced the swimming longevity, and concentrations close to 1 mM caused rapid encystment or death of the zoospores.

Absence of hyphal growth on polyamines as nitrogen sources. Radial growth from hyphal plugs transferred from V8 agar to YCA ceased after 12 days. Subculturing hyphal plugs to fresh V8 agar plates resulted in rapid growth, indicating that the hyphae were still viable. However, no growth occurred

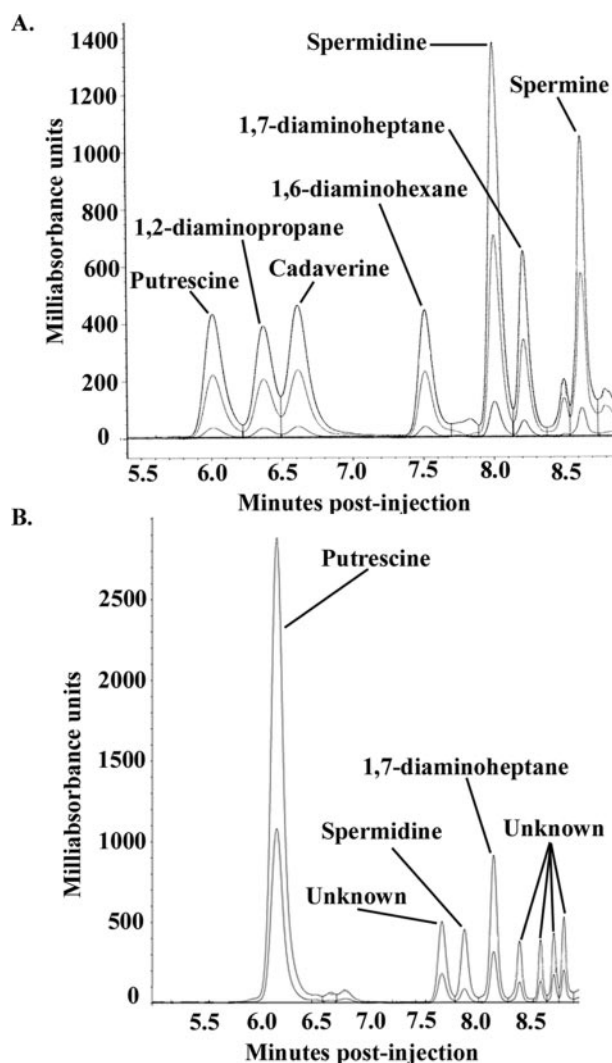


FIG. 1. Chromatograms of benzoylated polyamine standards (A) and benzoylated free polyamine extract from *P. sojae* hyphae (B). (A) Retention times and three absorbance profiles (240 nm [top curve], 250 nm [middle curve], and 270 nm [bottom curve]) are shown for each peak for 12 nmol benzoylated polyamine standards. (B) Putrescine from *P. sojae* hyphae eluted near 6 min and spermidine eluted at about 8 min. 1,7-Diaminoheptane, the internal standard, eluted after spermidine at about 8.2 min. The sample contained several unidentified compounds that produced peaks.

when hyphae were subcultured on YCA plates amended with putrescine, cadaverine, spermidine, or putrescine plus spermidine as the primary nitrogen source.

HPLC of polyamine standards. Polyamine standards had highly reproducible peaks with consistent retention times and peak areas when they were analyzed by HPLC. The retention times usually varied by no more than 4 s (but up to 15 s) over an 11-min separation. Polyamines eluted in order of carbon chain length except for putrescine (four C atoms), which eluted before 1,2-diaminopropane (three C atoms) (Fig. 1A). The peak heights for spermidine, a triamine, and the tetraamine spermine were greater than the peak heights for the diamines. Hyphal free polyamine extracts contained putrescine, spermidine, and some unidentified compounds.

TABLE 1. Endogenous putrescine and spermidine titers in *P. sojae* zoospores and hyphae

Source	Polyamine state	Concn (nmol/3 × 10 ⁵ zoospores) ^a		Concn (nmol/g [fresh wt] of hypha) ^b	
		Putrescine	Spermidine	Putrescine	Spermidine
Zoospores, 6 h	Free soluble	120 ± 10	10 ± 0.94		
	Both conjugated ^c	ND ^c	6.7 ± 0.84		
Zoospores, 36 h	Free soluble	78 ± 2.1	7.0 ± 0.57		
	Both conjugated	ND	9.0 ± 1.4		
Hyphae	Free soluble			230 ± 76	64 ± 46
	Soluble conjugated			38 ± 27	26 ± 23
	Insoluble conjugated			360 ± 170	92 ± 34

^a Zoospore concentrations of free and conjugated putrescine and spermidine were determined at two different times after zoospore emergence. In zoospores, soluble and insoluble conjugates were measured together. The values are means ± standard deviations.

^b Hyphal concentrations of free and conjugated putrescine and spermidine were determined. The values are means ± standard deviations.

^c ND, not detected.

Standard curves for polyamine quantification were highly reproducible with an R^2 value of >0.95 for each of the polyamine standards (data not shown). The presence of putrescine in hyphae and zoospore extracts and the presence of cadaverine in soybean roots were confirmed by gas chromatography-mass spectrometry.

Polyamines in soybean root tissue and exudate. Free putrescine, cadaverine, and spermidine were present at concentrations of 560 ± 260 , $5,300 \pm 1,500$, and 35 ± 9 nmol/g root tissue, respectively. Spermidine (17 ± 12 nmol/g) was the only polyamine detected in the soluble conjugate pool. Putrescine (10 ± 6.6 nmol/g) and cadaverine (83 ± 75 nmol/g), but not spermidine, were recovered from the insoluble conjugate pool of polyamines. Soybean roots released putrescine at a rate of 7.2 ± 1.2 nmol/g roots/h, but no other polyamines were detected in the exudates.

Putrescine in growth media. Putrescine was detected in V8 medium (61 ± 5.3 μ M), CV8 medium (32 ± 9.1 μ M), and various plate soak liquids. No other polyamines were identified in the medium samples.

P. sojae zoospores were produced by soaking V8 agar plates for 16 h, followed by repeated washing with water to stimulate sporangial production. After the 16-h soaking, liquid from a V8 agar plate with hyphae contained 7.2 μ M putrescine, while the solution overlying an uninoculated plate contained 12 μ M putrescine ($P < 0.01$, as determined by a two-tailed t test). After 8 h of repeated washing, the solution containing swimming zoospores typically contained <5 μ M putrescine.

Polyamine levels in zoospores and hyphae. *P. sojae* zoospores contained nanomoles of putrescine and spermidine per 3×10^5 cells (Table 1) but no detectable spermine or cadaverine. Zoospores contained approximately 10 times more free putrescine than free spermidine at both 6 and 36 h, no conjugated putrescine, and as much free spermidine as conjugated spermidine. As zoospores aged from 6 to 36 h postemergence, the free levels of both polyamines decreased by ~33%, but the conjugated levels of spermidine increased by ~25%. Hyphae grown in liquid CV8 medium contained 230 nmol/g (fresh weight) putrescine and ~60 nmol/g (fresh weight) spermidine (Table 1). Hyphae contained both soluble and insoluble conjugates of putrescine

and spermidine but lacked any detectable form of spermine or cadaverine. Bound putrescine occurred at a level that was ~1.5 times the level of free putrescine.

Uptake of polyamines by zoospores. Uptake of [1,4-¹⁴C]putrescine or spermidine by *P. sojae* zoospores increased linearly from 0 to 8 min. Preincubation of zoospores with unlabeled putrescine for 30 min prior to uptake of the label did not increase label uptake. The zoospores have a high-affinity, concentration-dependent putrescine transport system with Michaelis-Menten kinetics at concentrations from 3.75 μ M to 17.8 μ M, with an apparent K_m of 5.4 μ M and a V_{max} of 7.5 pmol/10⁵ zoospores/min. At substrate concentrations of >20 μ M, putrescine uptake did not exhibit classical Michaelis-Menten saturation kinetics. The zoospores also have a high-affinity concentration-dependent spermidine transport system with an apparent K_m of 4.6 μ M and a V_{max} of 2.4 pmol/10⁵ zoospores/min. At substrate concentrations of >30 μ M, spermidine uptake did not exhibit classical Michaelis-Menten saturation kinetics.

“Cross talk” between putrescine and spermidine transporters. The relationship between putrescine transport activity and spermidine transport activity in zoospores was assessed by allowing zoospores to take up 10⁵ dpm of radiolabeled putrescine or spermidine in the presence of excess amounts of each substrate (Table 2). Fifteen micromolar putrescine did not reduce the uptake of radiolabeled putrescine or spermidine compared to controls, while 15 μ M spermidine reduced the uptake of labeled spermidine but not the uptake of labeled putrescine. The combination of polyamines reduced putrescine uptake, and no spermidine uptake was detected. In the presence of either unlabeled substrate at a concentration of 100 μ M, labeled putrescine uptake was greatly reduced, and it was almost undetectable when each polyamine was added at a concentration of 100 μ M. One hundred micromolar unlabeled putrescine did not reduce the uptake of labeled spermidine, but 100 μ M unlabeled spermidine significantly reduced the uptake of labeled spermidine. Uptake of labeled spermidine was not detected when each polyamine was added at a concentration of 100 μ M. In a separate experiment 25 μ M cadaverine reduced the uptake of radiolabeled putrescine by $58\% \pm 20\%$.

TABLE 2. Relationship between putrescine transport activity and spermidine transport activity, expressed as the percentage of radiolabeled polyamine taken up in the presence of excess substrate^a

Nonradiolabeled polyamine(s) added	Radiolabel uptake (%)	
	Putrescine	Spermidine
No competitor	2.7 ± 0.71	2.9 ± 0.32
15 μM putrescine	2.4 ± 0.41	2.3 ± 1.1
15 μM spermidine	2.8 ± 0.53	0.32 ± 0.11
15 μM putrescine + 15 μM spermidine	0.87 ± 0.26	ND ^b
100 μM putrescine	0.25 ± 0.044	2.9 ± 0.22
100 μM spermidine	0.54 ± 0.21	0.15 ± 0.041
100 μM putrescine + 100 μM spermidine	0.022 ± 0.010	ND

^a Unlabeled putrescine and spermidine were added at concentrations of 0, 15, and 100 μM, either singly or together. Radiolabeled putrescine or spermidine (10⁵ dpm) was added to the reaction mixture, and uptake was measured. Excess spermidine inhibits both spermidine uptake and putrescine uptake, whereas excess putrescine does not appear to affect spermidine uptake. Significant differences among means were detected with the nonparametric Kruskal-Wallis test ($n = 3$).

^b ND, not detected.

DISCUSSION

In the present study we found that *P. sojae* zoospores have high-affinity, concentration-dependent putrescine transporters. The K_m that we observed is similar to the K_m values for putrescine transporters in yeast, *Leishmania*, *Escherichia coli*, *Trypanosoma cruzi*, rat aorta, and *Dictyostelium* (4, 10, 29, 35, 51, 52). Zoospores had a similar affinity for spermidine, although the V_{max} for putrescine was approximately three times higher than the V_{max} for spermidine. Incubation of zoospores in the presence of cadaverine reduced the uptake of labeled putrescine by 58%, suggesting that cadaverine also could be a substrate for the transporter(s) expressed in zoospores. A transporter for cadaverine may be important for *P. sojae* because this organism colonizes soybean roots, which contain high levels of cadaverine (25; this study).

At least two polyamine transporters, including putrescine and spermidine transporters, have been identified in several eukaryotic cell types (24, 50). We think that *P. sojae* has at least two transporters as well. Putrescine had minimal effects on spermidine uptake, but unlabeled spermidine reduced the uptake of labeled spermidine, so zoospores probably express a spermidine-specific transporter. Putrescine uptake was not reduced by 15 μM spermidine and was just barely detected in the presence of 100 μM spermidine. These observations are consistent with the existence of a second transporter that is down-regulated by spermidine or a transporter that preferentially, but competitively, transports putrescine.

The zoospore is a distinct stage in the life cycle of oomycetes, and genes encoding polyamine transporters may be part of the set of developmentally regulated genes that are typically expressed by zoospores in the soil. The turnover of organic matter in the soil ensures that polyamines are at least transiently available to swimming zoospores, and zoospores released from sporangia on infected soybean roots also should have access to polyamine-rich resources. It should be noted that the zoospores in this study were prepared in media rich in

polyamines, so transporter expression may have been induced by the polyamines present.

The growth media that we used to maintain *P. sojae* cultures contained free putrescine but no other polyamines. The level of putrescine in the V8 medium (61 μM) decreased by 50% as it was processed into CV8 medium (32 μM), due to removal of plant matter by centrifugation with CaCO₃. Hyphal V8 agar plates soaked with water (which leached polyamines) for 16 h to initiate zoospore production contained 7.2 μM putrescine, whereas uninoculated control plates contained 12 μM putrescine. The difference suggests that vegetative hyphae effectively scavenge putrescine from media during growth. During the course of infection, hyphae release necrosis-inducing peptides (42). We surmise that polyamine transporters are among a larger set of membrane transporters expressed to assimilate nutrients from host tissues. Thus, during three distinct developmental stages in the life history of oomycetes (motile zoospores, vegetative hyphae, and host infection) it seems likely that one or more polyamine uptake transporters are expressed.

Significant quantities of free and conjugated polyamines were detected in both zoospores and hyphae of *P. sojae*. The zoospores contained nanomole levels of putrescine and spermidine but lacked spermine and cadaverine (Table 1), and at 36 h zoospores contained ~33% less free putrescine and spermidine and ~25% more conjugated spermidine than zoospores contained at 6 h after emergence. Putrescine could be used for spermidine biosynthesis through the addition of an ethylamine residue to putrescine, since spermidine was not present in the zoospore media. Although the zoospores lacked detectable conjugated putrescine, they contained similar amounts of free spermidine and conjugated spermidine.

Hyphae contained substantial amounts of polyamines when they were grown in liquid CV8 medium (Table 1), and they had a lower ratio of free putrescine to free spermidine (4:1) than zoospores had (11:1). Hyphae contained both soluble and insoluble conjugates of putrescine and spermidine but no free or conjugated spermine or cadaverine. Bound (insoluble conjugated) putrescine was present at a level that was about 1.5 times the level of free putrescine. This finding is in stark contrast to the data for zoospores, which contained no detectable putrescine conjugates. In plant cell walls, polymerization of polyamines to other cell wall components is thought to contribute to strengthening of the cell wall during lignification (18). Localization of bound insoluble polyamines in zoospores or hyphae has not been established, but it is possible that polyamines contribute to strengthening of the hyphal cell wall as well. Thus, additional polyamines accumulated by swimming zoospores could support hyphal growth. Polyamines also may be critical for other aspects of hyphal growth prior to infection. In other eukaryotes high levels of polyamines are needed for cell division (16, 36, 49), and the shift from zoospores to hyphal germlings is associated with multiple nuclear divisions.

Despite the extensively documented uptake of spermidine (30, 32, 41, 53) and putrescine (5, 6, 10, 14, 47, 52, 53) and the myriad physiological roles of polyamines (7, 16, 31, 36), polyamines are not a major source of metabolic carbon or nitrogen for other organisms (1), and our observations suggest that this is also the case for oomycetes. However, plugs of hyphae subcultured from V8 agar to YCA could support growth to the perimeter of the plate in the absence of an N source, suggest-

ing that long-distance nutrient transport can facilitate hyphal movement through the soil.

Soybean roots contained more cadaverine than other polyamines, which is consistent with previous reports (25). We also estimated the levels of soluble and insoluble polyamine conjugates because in some tissues these conjugates account for a substantial proportion of the total polyamine pool (33, 45). However, in young root tissues, such conjugates represent only a small fraction of the total polyamine pool. Even though there were much higher internal levels of cadaverine, only putrescine root efflux was detected. Putrescine was also the only polyamine detected in tomato root exudates (34). In these exudates, the putrescine levels were 15 times higher than the levels reported for glutamic acid, the most abundant amino acid found in root exudates. The excretion of significant amounts of a nitrogen-rich compound is significant because nitrogen usually is a growth-limiting nutrient for plants and may have a higher "value" to the plant than photosynthetically derived compounds have.

A search of the draft sequence of the *P. sojae* genome (<http://phytophthora.vbi.vt.edu/>) identified 96 proteins with InterPro identifiers IPR002422 or IPR002293 for members of the polyamine/amino acid transporter families. A similar search of the *Arabidopsis* database (<http://www.arabidopsis.org/>) identified 75 proteins. A search for the predicted amino acid/polyamine transporters in *P. sojae* with the EST database (<http://www.pfgd.org>) identified five transcripts expressed by swimming zoospores. Notably, one of the genes expressed by swimming zoospores exhibits 22% identity and 40% similarity to LmPOT1 from *Leishmania major* (26). LmPOT1 is the only plasma membrane-localized putrescine-preferential transporter described to date for any eukaryote. Functional analysis of the zoospore homologue is in progress.

The polyamine transporters described here are only one aspect of the complex role that polyamines play in the life history of *P. sojae*. The changes in soluble and bound polyamines may mirror the changes reported for different developmental stages of other organisms (7, 45), and given that inhibition of polyamine metabolism can limit hyphal growth and alter the course of infection (9, 44), a better understanding of polyamine physiology in plants and oomycetes may result in identification of one or more unique targets for pathogen control.

ACKNOWLEDGMENT

We thank Jędrzej Romanowicz, Bowling Green State University, for technical assistance with gas chromatography-mass spectrometry.

REFERENCES

- Altman, A., and N. Levin. 1993. Interactions of polyamines and nitrogen nutrition in plants. *Physiol. Plant.* **89**:653–658.
- Antognoni, F., S. Fornale, C. Grimmer, E. Komor, and N. Bagni. 1998. Long-distance translocation of polyamines in phloem and xylem of *Ricinus communis* L. plants. *Planta* **204**:520–527.
- Antognoni, F., R. Pistocchi, P. Casali, and N. Bagni. 1995. Does calcium regulate polyamine uptake in carrot protoplasts? *Plant Physiol. Biochem.* **33**:701–708.
- Aouida, M., A. Leduc, R. Poulin, and D. Ramotar. 2005. *AGP2* encodes the major permease for high affinity polyamine import in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **280**:24267–24276.
- Badini, L., R. Pistocchi, and N. Bagni. 1994. Polyamine transport in the seaweed *Ulva rigida* (Chlorophyta). *J. Phycol.* **30**:599–605.
- Bagni, N., and R. Pistocchi. 1985. Putrescine uptake in *Saintpaulia* petals. *Plant Physiol.* **77**:398–402.
- Bais, H. P., and G. A. Ravishankar. 2002. Role of polyamines in the ontogeny of plants and their biotechnological applications. *Plant Cell Tissue Organ Cult.* **69**:1–34.
- Barash, I., J. M. Klisiewicz, and T. Kosuge. 1965. Utilization of carbon compounds by zoospores of *Phytophthora drechsleri* and their effect on motility and germination. *Phytopathology* **55**:1257–1261.
- Barker, J. H. A., T. A. Smith, and W. J. Owen. 1993. Inhibition of polyamine metabolism in *Phytophthora* species. *Mycol. Res.* **97**:1358–1362.
- Basselini, M., G. H. Coombs, and M. P. Barrett. 2000. Putrescine and spermidine transport in *Leishmania*. *Mol. Biochem. Parasitol.* **109**:37–46.
- Bimpong, C. E. 1975. Changes in metabolic reserves and enzyme activities during zoospore motility and cyst germination in *Phytophthora palmivora*. *Can. J. Bot.* **53**:1411–1416.
- Bimpong, C. E., and C. J. Hickman. 1975. Ultrastructural and cytochemical studies of zoospores, cysts, and germinating cysts of *Phytophthora palmivora*. *Can. J. Bot.* **53**:1310–1327.
- Caffaro, S., S. Scaramagli, F. Antognoni, and N. Bagni. 1993. Polyamine content and translocation in soybean plants. *J. Plant Physiol.* **141**:563–568.
- Calonge, M., J. C. Cubria, R. BalanaFouce, and D. Ordonez. 1996. Putrescine active uptake system in the trypanosomatid *Crithidia fasciculata*. *Biol. Chem. Hoppe-Seyler* **377**:233–238.
- Carlile, M. J. 1983. Motility, taxis and tropism in *Phytophthora*, p. 95–108. In D. C. Erwin, S. Bartnicki-Garcia, and T. H. Tsao (ed.), *Phytophthora: its biology, taxonomy, ecology and pathology*. APS Press, St. Paul, Minn.
- Cohen, S. S. 1998. A guide to the polyamines. Oxford University Press, New York, N.Y.
- Erwin, D. C., and O. K. Ribeiro. 1996. *Phytophthora* diseases worldwide. APS Press, St. Paul, Minn.
- Facchini, P. J., J. Hagel, and K. G. Zulak. 2002. Hydroxycinnamic acid amide metabolism: physiology and biochemistry. *Can. J. Bot.* **80**:577–589.
- Flores, H. E., and A. W. Galston. 1982. Analysis of polyamines in higher plants by high performance liquid chromatography. *Plant Physiol.* **69**:701–706.
- Fornale, S., T. Sarjala, and N. Bagni. 1999. Endogenous polyamine content and metabolism in the ectomycorrhizal fungus *Paxillus involutus*. *New Phytol.* **143**:581–587.
- Friedman, R., N. Levin, and A. Altman. 1986. Presence and identification of polyamines in xylem and phloem exudates of plants. *Plant Physiol.* **82**:1154–1157.
- Fujihara, S., H. Abe, Y. Minakawa, S. Akao, and T. Yoneyama. 1994. Polyamines in nodules from various plant-microbe symbiotic associations. *Plant Cell Physiol.* **35**:1127–1134.
- Fujihara, S., H. Abe, and T. Yoneyama. 1995. A new polyamine 4-aminobutylcadaverine occurrence and its biosynthesis in root-nodules of adzuki bean plant *Vigna angularis*. *J. Biol. Chem.* **270**:9932–9938.
- Fukumoto, G. H., and C. V. Byus. 1996. A kinetic characterization of putrescine and spermidine uptake and export in human erythrocytes. *BBA Biomembranes* **1282**:48–56.
- Gamarnik, A., and R. B. Frydman. 1991. Cadaverine, an essential diamine for the normal root development of germinating soybean (*Glycine max*) seeds. *Plant Physiol.* **97**:778–785.
- Hasne, M.-P., and B. Ullman. 2005. Identification and characterization of a polyamine permease from the protozoan parasite *Leishmania major*. *J. Biol. Chem.* **280**:15188–15194.
- Igarashi, K., K. Ito, and K. Kashiwagi. 2001. Polyamine uptake systems in *Escherichia coli*. *Res. Microbiol.* **152**:271–278.
- Igarashi, K., and K. Kashiwagi. 1999. Polyamine transport in bacteria and yeast. *Biochem. J.* **344**:633–642.
- Igarashi, K., and K. Kashiwagi. 1996. Polyamine transport in *Escherichia coli*. *Amino Acids* **10**:83–97.
- Kakinuma, Y., N. Masuda, and K. Igarashi. 1992. Proton potential-dependent polyamine transport by vacuolar membrane vesicles of *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1107**:126–130.
- Karigiannis, G., and D. Papaioannou. 2000. Structure, biological activity and synthesis of polyamine analogues and conjugates. *Eur. J. Org. Chem.* **2000**:1841–1863.
- Kashiwagi, K., R. Pistocchi, S. Shibuya, S. Sugiyama, K. Morikawa, and K. Igarashi. 1996. Spermidine-preferential uptake system in *Escherichia coli*—identification of amino acids involved in polyamine binding in PotD protein. *J. Biol. Chem.* **271**:12205–12208.
- Kotzabasis, K., and H. Senger. 1994. Free, conjugated and bound polyamines during the cell cycle in synchronized cultures of *Scenedesmus obliquus*. *Z. Naturforsch. Sect. C* **49**:181–185.
- Kuiper, I., I. Kuiper, G. V. Bloemberg, S. Noreen, J. E. Thomas-Oates, and B. J. J. Lugtenberg. 2001. Increased uptake of putrescine in the rhizosphere inhibits competitive root colonization by *Pseudomonas fluorescens* strain WCS365. *Mol. Plant-Microbe Interact.* **14**:1096–1104.
- Le Quesne, S. A., and A. H. Fairlamb. 1996. Regulation of a high-affinity diamine transport system in *Trypanosoma cruzi* epimastigotes. *Biochem. J.* **316**:481–486.
- Medina, M. A., J. L. Urdiales, C. Rodriguez-Caso, F. J. Ramirez, and F. Sanchez-Jimenez. 2003. Biogenic amines and polyamines: similar biochem-

- istry for different physiological missions and biomedical applications. *Crit. Rev. Biochem. Mol. Biol.* **38**:23–59.
37. Morel, M., C. Jacob, A. Kohler, T. Johansson, F. Martin, M. Chalot, and A. Brun. 2005. Identification of genes differentially expressed in extraradical mycelium and ectomycorrhizal roots during *Paxillus involutus-Betula pendula* ectomycorrhizal symbiosis. *Appl. Environ. Microbiol.* **71**:382–391.
 38. Morris, P. F., E. Bone, and B. M. Tyler. 1998. Chemotropic and contact responses of *Phytophthora sojae* hyphae to soybean isoflavonoids and artificial substrates. *Plant Physiol.* **117**:1171–1178.
 39. Morris, P. F., and E. W. B. Ward. 1992. Chemoattraction of zoospores of the soybean pathogen, *Phytophthora sojae*, by isoflavones. *Physiol. Mol. Plant Pathol.* **40**:17–22.
 40. Ozawa, T., and T. Tsuji. 1993. A possible role for polyamines in the repression of growth of *Bradyrhizobium japonicum* bacteroids in soybean nodules. *Plant Cell Physiol.* **34**:899–904.
 41. Pistocchi, R., F. Antognoni, N. Bagni, and D. Zannoni. 1990. Spermidine uptake by mitochondria of *Helianthus tuberosus*. *Plant Physiol.* **92**:690–695.
 42. Qutob, D., S. Kamoun, and M. Gijzen. 2002. Expression of a *Phytophthora sojae* necrosis-inducing protein occurs during transition from biotrophy to necrotrophy. *Plant J.* **32**:361–373.
 43. Redmond, J. W., and A. Tseng. 1979. High-pressure liquid chromatographic determination of putrescine, cadaverine, spermidine and spermine. *J. Chromatogr.* **170**:479–481.
 44. Ross, W. F., D. R. Walters, and D. J. Robins. 2004. Synthesis and antifungal activity of five classes of diamines. *Pest Manag. Sci.* **60**:143–148.
 45. Saran, S. 1998. Changes in endogenous polyamine levels are associated with differentiation in *Dictyostelium discoideum*. *Cell Biol. Int.* **22**:575–580.
 46. Schmitthenner, A. F. 1985. Problems and progress in control of *Phytophthora* root-rot of soybean. *Plant Dis.* **69**:362–368.
 47. Seiler, N., J. G. Delcros, and J. P. Moulinoux. 1996. Polyamine transport in mammalian cells. An update. *Int. J. Biochem. Cell Biol.* **28**:843–861.
 48. Simpson, A. G. B., and A. J. Roger. 2004. The real “kingdoms” of eukaryotes. *Curr. Biol.* **14**:R693–R696.
 49. Slocum, R. D., R. Kaursawhney, and A. W. Galston. 1984. The physiology and biochemistry of polyamines in plants. *Arch. Biochem. Biophys.* **235**:283–303.
 50. Tomitori, H., K. Kashiwagi, T. Asakawa, Y. Kakinuma, A. J. Michael, and K. Igarashi. 2001. Multiple polyamine transport systems on the vacuolar membrane in yeast. *Biochem. J.* **353**:681–688.
 51. Toursarkissian, B., E. D. Endean, and S. M. Aziz. 1994. Characterization of polyamine transport in rat aortic smooth muscle cells. *J. Surg. Res.* **57**:401–407.
 52. Turner, R., M. J. North, and J. M. Harwood. 1979. Putrescine uptake by the cellular slime mould *Dictyostelium discoideum*. *Biochem. J.* **180**:119–127.
 53. West, H. M., and D. R. Walters. 1991. Polyamine uptake by the plant pathogenic fungus, *Fusarium culmorum*. *Mycol. Res.* **95**:715–719.
 54. Young, C. C., and L. F. Chen. 1997. Polyamines in humic acid and their effect on radical growth of lettuce seedlings. *Plant Soil* **195**:143–149.