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# Vascular blackening of wasabi rhizomes caused by *Pectobacterium carotovorum* subsp. *carotovorum*

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**Abstract** Wasabi (*Wasabia japonica*) is grown for its highly-valued rhizome which is used as a condiment in Japanese food. Symptoms of vascular blackening in the rhizome were first observed in 2005 in plants grown in British Columbia, Canada. Microscopic observations and microbial isolation from infected tissues revealed that most of the xylem tracheid cells were blackened and bacteria were consistently associated with symptomatic plants. The bacterium most frequently recovered was identified as *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) using BioLog™ and sequencing of a specific ~510 bp IGS region. Pathogen-free plants obtained using meristem-tip micropropagation were inoculated with a wasabi isolate of *Pcc*. Vascular blackening symptoms developed in the rhizome after 8 weeks when the rhizome was first wounded by stabbing or cutting, or if the roots were pre-inoculated with *Pythium* species isolated from rhizome epidermal tissues, followed by inoculation with *Pcc* at $1\times10^8$ cells $ml^{-1}$. Xylem tracheid cells were blackened and *Pcc* was reisolated from all diseased tissues. The highest frequency of rhizome vascular blackening occurred at 22°C and 27°C and these tissues occasionally succumbed to soft rot at higher temperatures, but not when inoculated tissues were incubated at 10°C. The rooting medium used by growers for vegetative propagation of wasabi was shown to contain *Pcc* but the pathogen was not recovered from the irrigation water. Entry of *Pcc* through wounds on wasabi rhizomes and the host tissue response result in symptoms of vascular blackening.

**Keywords** Histopathology · *Wasabi japonica* · *Pythium* spp. · Wounding

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## Introduction

Wasabi (*Wasabia japonica*) is a perennial plant which is native to Japan, where the freshly-ground rhizome has been eaten as a condiment since the 10th century (Chadwick et al. 1993). Wasabi can be grown in soil but high-quality rhizomes for the fresh market are traditionally cultivated in stream beds or in hydroponic or semi-hydroponic conditions. Good quality rhizome production requires water with a high level of dissolved oxygen and the plant grows best at air temperatures between 8°C to 18°C and growth ceases above 28°C (Follet 1986). To initiate new plantings of wasabi, axillary shoots with subtending leaves are used for vegetative propagation. This method is widely used by growers since establishing new plants from seeds is difficult because of an extended seed

dormancy period, a low germination rate, and high seedling mortality. However, continuous vegetative propagation of wasabi can result in a gradual decline in plant growth rate (Chadwick et al. 1993), and also allows pathogens to build up, thereby reducing plant survival and potentially spreading pathogens.

A major factor which reduces wasabi rhizome quality is a visually unappealing external or internal blackening of the rhizome (Follet 1986; Goto and Matsumoto 1986; Lo et al. 1990). These rhizomes are often sold for processing instead of for the fresh market or are discarded, and severely affected fields may be abandoned by growers (Chadwick et al. 1993). Rhizome-blackening symptoms appear to be the result of an accumulation of phenolic compounds in tissues damaged by biotic agents or stress factors. Blackening of wasabi rhizomes has been reported from various regions where the crop is cultivated, including New Zealand (Broadhurst and Wright 1998), Australia (Sparrow 2006) and Taiwan (Lo and Wang 2000a). The symptoms may develop in various tissues, including the epidermis, cortex, vascular tissue and/or pith of the rhizome (Goto and Matsumoto 1986; Adachi 1987; Chadwick et al. 1993; Sparrow 2006). These blackening symptoms have been previously referred to as blackleg (Chadwick et al. 1993), streak disease (Lo et al. 1990; Wang et al. 1992), internal black rot syndrome (Goto and Matsumoto 1986), or black rot disease (Lo and Wang 2000a). Much of the blackening symptoms on the rhizome surface and on leaves and petioles has been attributed to the Ascomycete *Phoma wasabiae* (Goto and Matsumoto 1986; Adachi 1987; Lo et al. 1990; Wang et al. 1992; Chadwick et al. 1993; Lo and Wang 2000b; Sparrow 2006). However, internal blackening of the rhizome, mostly in the vascular tissues, appears to be a previously unreported symptom with no known cause or etiology.

Wasabi is currently grown in British Columbia (B. C.), Canada, and is a recently introduced crop. Symptoms of blackened vascular tissues in rhizomes of cvs Daruma and Mazuma were observed during 2005. The symptoms were unique from those previously reported in the literature, in that externally healthy-appearing rhizomes showed internal vascular blackening that significantly reduced their marketability as a fresh condiment. The objectives of this research were to recover and identify microbes from affected rhizomes and to complete Koch's postulates to establish the cause of internal blackening.

## Materials and methods

### Microbial isolation

Wasabi cv. Daruma plants (Fig. 1a) grown in Richmond, B.C. were collected approximately every 10 weeks from November, 2005 to August, 2006. A total of about 100 plants were sampled. Those with internal vascular blackening (about 60%) were identified by slicing into the rhizome (Fig. 1b, c). In the majority of these rhizomes, there were no external symptoms of blackening. Leaves were trimmed off and the upper and lower 1 cm of the affected rhizome was discarded. The remainder of the rhizome was rinsed in running water for 15 min. Sections (2 $mm^3$) taken from regions representing the epidermis, cortex, vascular and pith (Fig. 1d) were individually surface-sterilised in 70% ethanol for 30 s followed by 0.25% NaOCl for 1 min and plated onto water agar (WA, Anachemia) containing 300 mg $l^{-1}$ streptomycin sulphate (Sigma-Aldrich) or onto nutrient agar (NA, Difco) without antibiotics. The dishes were incubated at room temperature (21°C–23°C) for 1–2 weeks. Fungi recovered on WA were transferred to potato dextrose agar (PDA) and identified to genus level using morphological criteria. Bacteria recovered on NA were grouped as Gram-positive or Gram-negative according to the KOH method (Fluharty and Packard 1967). Morphologically distinct colonies of Gram-negative bacteria were identified using Biolog™ (Biolog Inc., USA). Cultures were stored in 80% glycerol at −80°C.

### Microscopic observations

For light microscopic observations, small tissue segments were taken from eight different wasabi rhizomes with symptoms of vascular blackening (Fig. 1d), fixed in formaldehyde–acetic acid (FAA) for 48 h and rinsed in 70% ethanol. Sections (3 μm thick) were cut, immediately stained with 0.1% toluidine blue for 30 s and examined under a light microscope at various magnifications. The presence or absence of fungal hyphae and bacterial cells was noted for each sample.

### Pathogenicity tests

Select bacterial isolates representing each bacterial species were first tested for pathogenicity on cabbage

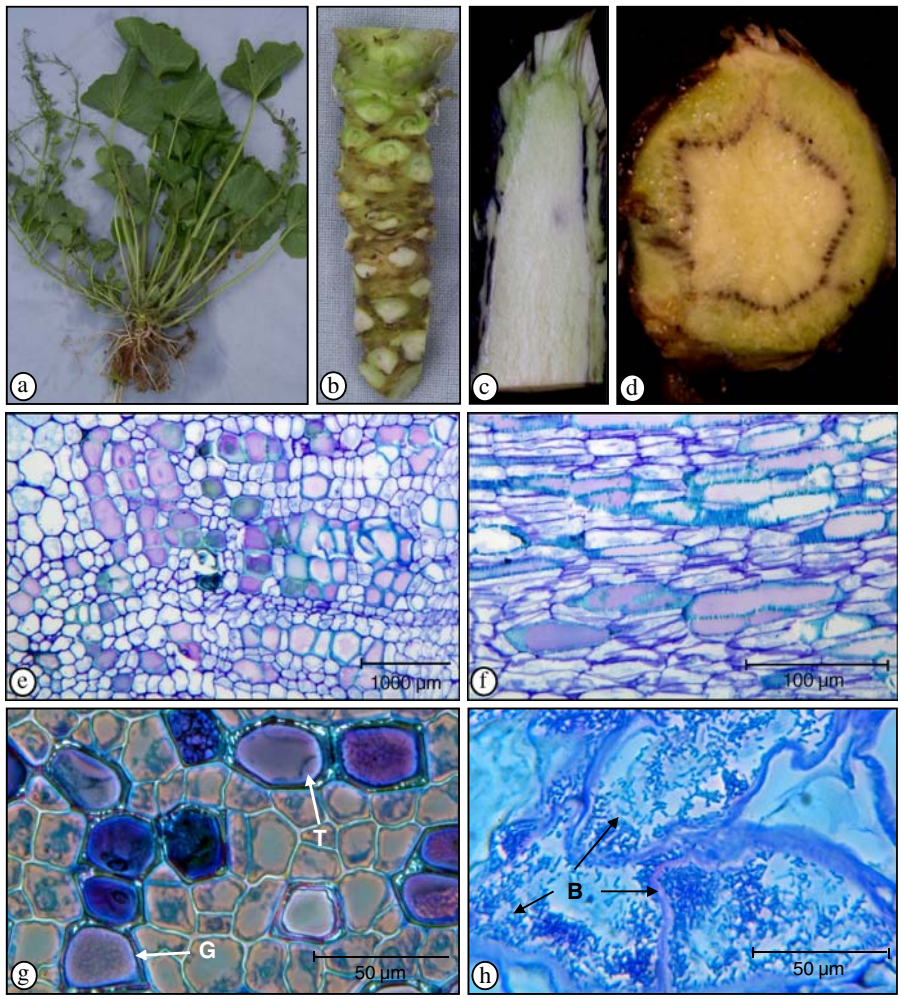


**Fig. 1** Wasabi rhizomes with vascular blackening. **a** Healthy plants; **b** healthy rhizome; **c**, **d** plants with symptoms of vascular blackening in **c** longitudinal section and **d** in cross-section. **e**–**h** Microscopic images of wasabi rhizome showing rhizome vascular blackening. **e** Cross-section of blackened rhizome vascular tissue stained with toluidine blue; **f** as in (**e**), but longitudinal section. **g** Cross-section of affected tissue under phase-contrast illumination; gels (*G*) and tylose (*T*). **h** Close-up of xylem parenchyma cells with bacteria (*B*)

leaves. A bacterial suspension in PBS buffer ($10^8$ cells $ml^{-1}$) of each species was injected into the mesophyll space of the leaf using a syringe. Control leaves received PBS buffer only. The development of necrotic areas was rated after 2 days. For isolates identified as *Pectobacterium carotovorum*, pectolytic enzyme activity was determined according to Schaad (1988). Slices of potato cv. Yukon Gold (purchased at a local store) were inoculated with bacterial suspensions ($10^4$–$10^8$ cells $ml^{-1}$ suspended in PBS). For wasabi inoculations, fresh rhizomes, sliced longitudinally, were surface-sterilised and similarly inoculated with *P. carotovorum* and a number of other representative bacterial species. Tissues were placed inside plastic containers lined with moist filter paper under ambient conditions and examined for evidence of decay after 48 h. The experiment was conducted twice.

A number of *Pythium* and *Fusarium* isolates were recovered from wasabi roots and tested for pathogenicity using tissue culture-derived plants (see below) following the hydroponic culture procedure described by Rodriguez and Punja (2007). Cultures were grown on PDA for 2 weeks and 5×5 mm agar plugs were added to the hydroponic solution. Development of root rot or blackening symptoms was assessed after 12 weeks at 22°C. There were ten plants for each isolate and the experiment was conducted twice. Pathogen re-isolation was conducted from three 1 cm long root segments after surface-sterilising them as described previously and plating onto WA with 300 mg $l^{-1}$ streptomycin sulphate.

## Tissue-cultured plant material

Wasabi plants cv. Daruma were obtained from a commercial greenhouse located in Richmond, B.C., the leaf petioles were trimmed from the rhizome to expose the axillary buds (Fig. 1b), and rinsed under running tap water for 5 min. The meristem region, measuring 1–1.5 mm, with approximately five leaf primordia, was excised from within the axillary bud and immersed in a 0.5% NaOCl solution containing Tween 20 for 15 min, rinsed four times in sterile distilled water (SDW), and finally soaked in SDW for 5 min. Meristems were placed in 60 mm diam Petri dishes containing Gamborg's B5 medium (Gamborg et al. 1968) with 0.5 mg $l^{-1}$ $N^6$-benzyladenine (BA) (Sigma-Aldrich), solidified with 2.2 g $l^{-1}$ phytagel (Sigma-Aldrich) and incubated on a laboratory bench (temperature was approximately 21°C– 23°C with a 16 h photoperiod, under a light intensity of 14 μmol $m^{-2}s^{-1}$ provided by white fluorescent tubes). After 10 days, meristems free of visible fungal or bacterial contamination and appeared healthy were transferred to 100 ml glass plant tissue culture vessels (Sigma-Aldrich) containing 20 ml of Gamborg's B5 medium (Gamborg et al. 1968) with 0.5 mg $l^{-1}$ BA solidified with 2.2 g $l^{-1}$ phytagel (Sigma-Aldrich). The vessels were placed inside a Percival growth chamber set at 16°C with a 16 h photoperiod and light intensity of 14 μmol $m^{-2}$ $s^{-1}$ provided by white fluorescent tubes. These meristems produced clusters of axillary shoots, which were aseptically separated every 2 months and transferred to fresh medium. To determine if these in vitro plants were free of microbial contamination, shoots were aseptically macerated using a mortar and pestle and suspended in phosphate-buffered (0.01 M, pH 7.1) saline (0.85%) solution (PBS) and 10 μl was plated onto NA and PDA and incubated under laboratory conditions for 2 weeks. If no bacterial or fungal growth occurred, the resulting plants were assumed to be free of associated microorganisms. Individual axillary shoots were then rooted on hormone-free Gamborg's B5 medium (Gamborg et al. 1968) solidified with phytagel (Sigma-Aldrich) in 77 mm×77 mm×97 mm Magenta® vessels (Sigma-Aldrich) placed inside a growth chamber under the conditions described previously. After 2 months, the plants were gently removed, the agar was washed off, and they were used for inoculation experiments.

## Induction of vascular blackening symptoms

The tissue-culture derived plants described above were used to determine whether *Pectobacterium* could induce rhizome vascular blackening. This bacterium was selected for further study for two reasons: it was the most frequently isolated bacterium from rhizome tissues (>70% frequency) and it was pathogenic on wasabi tissues in vitro causing tissue decay. Lateral roots on plants were trimmed and a small portion (2 mm) of the rhizome bottom was sliced off with a scalpel to create wounds and compared to unwounded plants. There were ten replicate plants for each treatment. Rhizomes were soaked in PBS containing $10^8$ cells $ml^{-1}$ of bacteria for 30 min and placed in potting mix used by commercial growers (100% steer manure, Home Depot, Coquitlam, B.C.) which was autoclaved twice prior to use. Controls included unwounded plants and wounded plants that received buffer only. All plants were grown for 8 weeks in a Percival growth chamber set at 22°C, 90% relative humidity and a 14 h photoperiod with a light intensity of 10 μmol $m^{-2}$ $s^{-1}$ provided by cool-white fluorescent lamps. Rhizomes were then harvested and sliced and examined for blackening symptoms, followed by microscopic observations as described previously. The experiment was conducted twice.

To determine if there was a potential interaction between *Pythium* species and *P. carotovorum*, wasabi plants were grown in a hydroponic system as described previously (Rodriguez and Punja 2007). Plugs from colonies of two *Pythium* species identified previously as *P. dissotocum* and *P. intermedium* (Rodriguez and Punja 2007) were added to the hydroponic solution and plants were grown at 18°C. There were ten plants per treatment. One week later, plants were inoculated with $10^8$ cells $ml^{-1}$ of bacteria by soaking the roots for 30 min. An additional set of plants was wounded by stabbing with a needle and inoculated with the bacteria. Control plants were similarly wounded and soaked in PBS, or not disturbed. The plants were returned to 22°C for 8 weeks and development of rhizome blackening was noted. The experiment was conducted twice.

## Effect of temperature on rhizome blackening

To determine how temperature affects blackening symptom development, rhizomes on tissue-cultured

plants were wounded by slicing as before, or stabbed with a dissecting needle, and compared to unwounded plants. There were ten plants per treatment and the experiment was conducted twice. Bacterial inoculation was conducted as described previously and plants were transferred to potting mix and grown at 22°C for 8 weeks. After this period, plants were transferred to 10, 22 or 27°C and incubated for an additional 8 weeks. The rhizomes were sliced and the presence or absence of blackened vascular tissue was assessed visually. For assessment of treatment effects, data were analysed by ANOVA and means separated using Fisher's LSD test. Bacterial isolation was attempted from asymptomatic and symptomatic plants (with blackening) after surface-sterilising rhizome sections and plating them onto NA. Isolated bacterial colonies were suspended in 9 ml PBS and 10 µl was pipetted and spread onto crystal violet pectate (CVP) medium in a 60 mm Petri dish (VWR) (Schaad 1988). Colonies exhibiting pectolytic activity were selected and picked with a sterile pipette tip and lysed in 5 µl of sterile water by placing at 95°C for 5 min. Identification using PCR was performed as described below.

## Identification of *Pectobacterium* subspecies

Growth of *P. carotovorum* isolates from wasabi was compared to that of known strains of *Pcc* and subsp. *wasabiae* (*Pcw*) (Table 1) (provided by S. H. De Boer, Canada Food Inspection Agency, Charlottetown). For the potassium cyanide (KCN) growth test (Goto and Matsumoto 1987), isolates were grown on NA and then suspended in tryptic soy broth (Difco) to 0.5 McFarlands (Cowan and Steel 1974; Goto and Matsumoto 1987). KCN broth or broth base was prepared according to Cowan and Steel (1974) and 3 ml aliquots were placed in 14 mm round bottom snap cap Falcon tubes (BD Biosciences). One microliter of suspended bacteria was added and 1 ml of sterile paraffin was pipetted on top to provide the anaerobic treatments, while the aerobic controls were left open to the air. The cultures were incubated at 30°C for 2 weeks.

Sequence comparison of the IGS region was also conducted. All isolates were grown for 2 days in tryptic soy broth and DNA was extracted according to the method of Lee et al. (1993). The previously published primers G1f: GAAGTCGTAACAAGGTA and L1r: CAAGGCATCCACCGT were used to amplify a region of the 16S–23S IGS rDNA (Jensen et al. 1993) according to Fessehaie et al. (2002) except with an annealing temperature of 55°C and 31 cycles. PCR reactions were performed in a GeneAmp PCR System 9700. Fifteen µl of product was run on a 2% agarose gel (BioShop) in a Tris–acetate–EDTA (TAE) buffer at 80 V for 1.5 h. The smaller DNA band (~510 bp) was obtained using the 'band stab' method (Wilton et al. 1997) with primers G1f and L1r, and sequenced by Macrogen Inc. (Seoul, South Korea) using the same primer set and compared to IGS sequences in GenBank corresponding to *Pcc* and *Pcw*.

## Identification of inoculum sources

The potting mix used by commercial growers for vegetative propagation of wasabi, which consists of composted manure, was sampled from three unused bags. One gram was placed in PBS and dilution-plated (up to $10^{-6}$) onto CVP plates. After 4 days, two colonies with pectolytic activity were selected from the $10^{-5}$ dilution plate, DNA was extracted and amplified by PCR as described previously. Water samples were taken from the sprinkler heads at the greenhouse in July, 2005 and February, 2006 and dilution-plated up to $10^{-6}$ on NA and PDA.

**Table 1** Reference strains of *Pcc* and *Pcw* used in this study

| Bacterium | Strain | Host of origin | Country of origin | GenBank accession number 16S/Small IGS |
|---|---|---|---|---|
| *Pcc* | 155 | Potato | Canada (B. Copeman) | AF373188 |
| *Pcc* | 517 | Potato | USA (M. Powelson) | – |
| *Pcw* | 91 | Wasabi | Japan (M. Goto) | AF373193 |
| *Pcw* | 92 | Wasabi | Japan (M. Goto) | AF373194 |

## Results

### Microbial isolation and light microscopy

Wasabi plants with internal vascular blackening appeared visibly healthy when viewed externally (Fig. 1a, b). From 60 symptomatic plants sampled (Fig. 1c, d), various bacterial colonies were recovered on NA at 100 and 80% frequency from the epidermis and vascular regions, respectively. The cortex and pith tissues did not yield any bacteria. Using Biolog™, the bacteria were identified as *Agrobacterium tumefaciens*, *Enterobacter intermedius*, *Pcc*, *Pseudomonas fluorescens*, and *Pseudomonas fulva* with a probability of 100, 100, 99, 96, and 97%, respectively. The most frequently recovered bacterium from wasabi rhizomes was *P. carotovorum* (>70% recovery). Other microbes recovered from wasabi rhizome tissues included the two *Pythium* species reported previously (Rodriguez and Punja 2007) as well as two isolates of *Fusarium sambucinum*. These microbes originated from epidermal tissues and not from the cortex, vascular region, or pith of symptomatic rhizomes.

Light microscopic examination of symptomatic tissues showed that the xylem tracheid cells were blackened (Fig. 1e, f). Gels and tyloses were observed in the xylem tracheids (Fig. 1g), indicative of host responses to systemic infection by microbes (Beckman 2000). Bacterial cells were often seen within and between parenchyma cells which were in close proximity to the blackened xylem tracheids (Fig. 1h). Only one of eight symptomatic samples examined microscopically did not contain visible bacteria, and bacteria were always visible in epidermal tissues. Mycelia were not observed near the vascular tissue of the symptomatic rhizomes in any sample; however, they were visible in epidermal tissues.

### Pathogenicity tests

No symptoms developed on any of the cabbage leaves inoculated with the different bacterial species (data not shown). Inoculation of *P. carotovorum* on potato and wasabi tissues resulted in tissue maceration within 48 h. None of the other bacterial species caused any symptoms. Potato tissue was degraded at concentrations $\geq 10^5$ cells $ml^{-1}$ of *P. carotovorum*, while wasabi maceration occurred at $\geq 10^7$ cells $ml^{-1}$. The two *Pythium* species caused significant lateral root loss and were successfully reisolated from diseased tissues. Black lesions developed on most plants at the base of petioles in contact with the hydroponic solution containing inoculum of *F. sambucinum*. However, there was no affect on root growth (data not shown), and *F. sambucinum* was not reisolated from root tissues. Vascular blackening did not develop on any of the rhizomes inoculated with either *Pythium* species or *F. sambucinum* (Table 2).

### Identification of *Pectobacterium* subspecies

The KCN test showed that the subspecies recovered from wasabi rhizomes was likely to be subsp. *carotovorum* and not subsp. *wasabiae*. The wasabi isolates and the known *Pcc* isolates (strains 155 and 157) grew under both aerobic and anaerobic conditions in the KCN broth, while the confirmed subsp. *wasabiae* isolates (strains 92 and 94) grew only under aerobic conditions. The identity of the wasabi isolates was confirmed when the IGS-PCR products were shown to consist of two bands of ~510 and ~550 bp, which is typical of *Pcc* subsp. *atroseptica*, and subsp. *betavasculorum* (Toth et al. 2001; Fessehaie et al. 2002). The smaller IGS-PCR band (~510 bp) was sequenced and when compared to other sequences in GenBank, it showed 99% homology with *Pcc* strain E161 (GenBank accession number AF373189.1) and 96% homology with *Pcw* strain ATCC 43316 (GenBank accession number AF232679).

### Induction of vascular blackening symptoms

Pathogen-free wasabi plants propagated in tissue culture (Fig. 2a, b), when inoculated with *P. carotovorum* developed blackening in the rhizome vascular tissue (Fig. 2d–f), despite appearing outwardly healthy (Fig. 2c). When the rhizome base was cut prior to inoculation, 70% of the rhizomes (out of ten plants) developed black streaks in the vascular tissue while 15% exhibited soft rot symptoms and died (Table 2). These data were consistent over the two repeated experiments conducted and the mean percent blackening was similar among all replications. None of the unwounded inoculated plants developed vascular blackening while one wounded uninoculated

**Table 2** Summary of pathogenicity tests conducted on wasabi plants using microbes isolated from blackened tissues

| Microbe tested | Treatment[a] | External symptoms[b] | Percentage of plants with vascular blackening[c] |
|---|---|---|---|
| *Pectobacterium carotovorum* | Wounded by cutting, inoculated | Soft rot in 10% of plants | 70 |
| | Wounded by stabbing, inoculated | None | 45 |
| | Not wounded, inoculated | None | 0 |
| *Pythium dissoctocum* and *P. intermedium* | Inoculated in hydroponic system | Root rot | 0 |
| *Fusarium sambucinum* | Inoculated in hydroponic system | Black lesions on petioles | 0 |
| *P. carotovorum* and *Pythium* spp. | *Pythium* inoculation followed by *P. carotovorum* after 1 week | Root rot | 45–55 |
| None | Wounded or not wounded | None | 0 |

[a] Following each treatment, plants were incubated at 22°C in a growth chamber for 8 weeks

[b] Plants were assessed visually for any external symptoms

[c] Rhizomes were sliced longitudinally and rated for presence or absence of vascular blackening. Data are from ten plants in each of two separate experiments

plant developed blackening in the vascular tissue. The pathogen was successfully reisolated from all symptomatic plants. While other bacterial colonies were recovered on NA from wounded uninoculated and healthy plants, they did not form pectolytic pits on CVP, indicating they were not *Pcc*. These bacteria were not identified further. Microscopic examination of blackened vascular tissues in samples artificially inoculated with *P. carotovorum* revealed blackened xylem tracheids (Fig. 2g–k) with bacterial cells present in close proximity to xylem parenchyma cells (Fig. 2h, j).

Plants wounded by stabbing or inoculated with *P. dissotocum* or *P. intermedium*, followed by inoculation with *P. carotovorum*, developed rhizome vascular blackening at a frequency of 45±8%, 55±7%, and 40 ±10%, respectively (data from two experiments) (Table 2). Control plants wounded or not wounded and did not receive bacteria, or plants inoculated with *Pythium* species alone, were asymptomatic.

### Effect of temperature on rhizome blackening

Wasabi rhizomes wounded by cutting off the base and inoculated with *Pcc* followed by 8 weeks of incubation at 10, 22 or 27°C showed vascular blackening in 55–87% of the plants at the three temperatures (Fig. 3). Soft rot developed in 8–10% of the inoculated plants that were incubated at 22 or 27°C but not at 10°C. Blackened petioles and leaf veins were observed on plants incubated at 27°C. Plants wounded by stabbing and inoculated with *Pcc* developed less symptoms (25–40%) compared to those wounded by cutting the rhizomes (Fig. 3). Control plants wounded only and not inoculated did not develop any symptoms.

### Identification of inoculum source

Colony-IGS-PCR conducted on two bacterial isolates recovered from potting mix showed the same banding pattern as that of *P. carotovorum* from rhizomes (Fig. 3), and there was 100% homology in the ~510 bp IGS region with that of a *Pcc* isolate obtained from blackened rhizome vascular tissues on greenhouse-grown wasabi plants (Fig. 4). No bacteria or fungal colonies were recovered from water samples obtained from the irrigation heads in the greenhouse.

## Discussion

Wasabi rhizomes with internal vascular blackening symptoms yielded a high frequency of recovery of *Pcc* after surface-sterilisation of tissues. Inoculation of tissue-culture derived plants with this bacterium produced symptoms identical to those seen on greenhouse-grown plants, both macroscopically and microscopically. The pathogen was also successfully reisolated from inoculated plants and identified. In

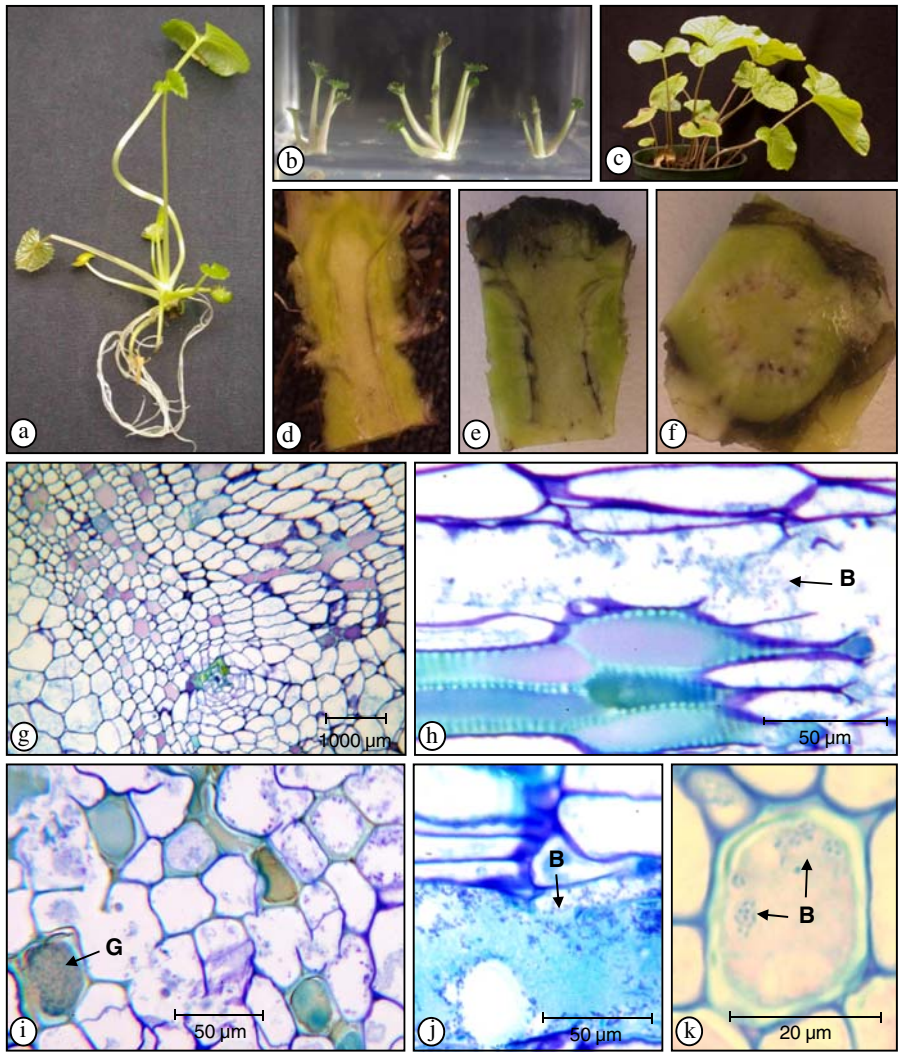


**Fig. 2** Reproduction of wasabi vascular blackening symptoms. **a**, **b** Meristem-tip derived pathogen-free tissue culture plants inoculated with *Pcc*. **c** Whole plant 8 weeks after inoculation. **d**, **e**, **f** Rhizomes in which plant roots were stabbed (**d**) or cut (**e**, **f**) and then inoculated with *Pcc*. Blackened vascular tissues can be seen. **g**–**k** Microscopic images of blackened vascular rhizome tissue depicting gels (*G*) and bacteria (*B*) in xylem parenchyma (**h**, **j**) and xylem tracheid (**k**) cells, following artificial inoculation

diseased tissues, blackened xylem tracheids in the rhizome were surrounded by xylem parenchyma cells which contained bacteria. The tissue blackening symptom in wasabi is presumed to be due to accumulation of oxidised phenolic compounds produced as a host defence response (Mace et al. 1972; Matsuki 1996; Ploetz 2005) and is likely to be a general response to microbial invasion. A similar blackening of vascular tissues is seen on potato infected by *Pcc* causing blackleg (Pérembelon 2002).

While *P. wasabiae* is reported to cause blackening of the rhizome surface, leaves and petioles of wasabi (Goto and Matsumoto 1986; Adachi 1987; Lo et al. 1990; Wang et al. 1992; Chadwick et al. 1993; Lo and Wang 2000b; Sparrow 2006), this pathogen has not been isolated from blackened wasabi rhizomes grown in B.C. In New Zealand, *P. wasabiae* was isolated from diseased rhizomes in only nine out of 192 plants, (Broadhurst and Wright 1998) while Goto and Matsumoto (1986) reported that only 14 out of 149 plants with rhizome vascular blackening symptoms yielded *P. wasabiae*.

Previous studies from Japan (Goto and Matsumoto 1986) and New Zealand (Broadhurst and Wright 1998) reported the recovery of a range of bacterial species, including *P. carotovorum* and *Erwinia* species, as well as *Pseudomonas* species, from blackened wasabi tissues. However, inoculation experiments were not conducted with these bacteria and therefore their role in wasabi rhizome-blackening is unknown. Bacteria identified as *Pseudomonas* species and *Agrobacterium tumefaciens* and *Enterobacter inter-*

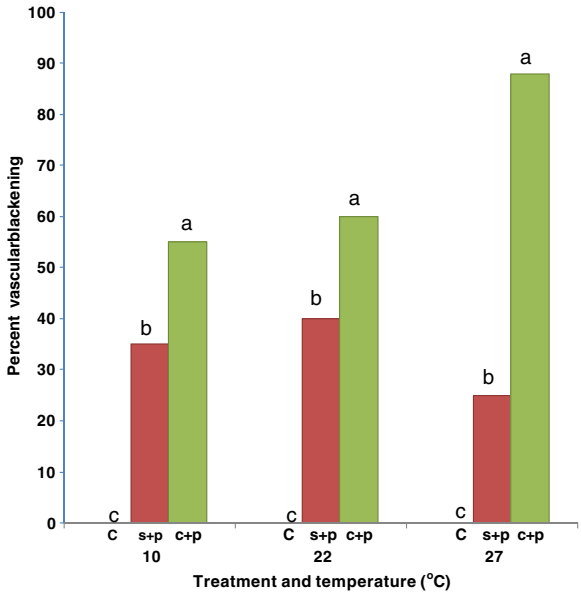


**Fig. 3** Incidence of rhizome vascular blackening in wasabi plants inoculated with *Pcc* following wounding and grown in potting mix at different temperatures for 8 weeks. Data are from two separate experiments combined ($n$=20). *C* control (no wounding or inoculation); *S*+*P* stabbing plus inoculation; *C*+*P* cutting plus inoculation. *Columns followed by the same letter* within each temperature are not significantly different according to Fishers protected LSD test ($P$=0.05)

*medius* were recovered at a low frequency (5–18%) from symptomatic rhizomes in the present study. These bacteria may be invaders of diseased tissues or occur as saprophytes colonising the surface of wasabi plants, since none were shown to be pathogenic.

Wounds created by cutting the rhizome followed by inoculation with *P. carotovorum* caused greater vascular blackening symptoms than stabbing, which may not have provided sufficient entry points into the vascular tissues. While *P. carotovorum* primarily caused rhizome vascular blackening, soft rot of tissues was occasionally observed at higher temperatures (plants grown at 27°C) in potting mix with high moisture content. These conditions are conducive to the development of *P. carotovorum*, an opportunistic pathogen and a facultative anaerobe (Pérembelon 2002).

On potato, *P. carotovorum* is often associated with asymptomatic plants and induces symptoms once conditions become favourable or host resistance is impaired (Pérembelon 2002). Growth of the pathogen occurs at temperatures around 27°C and when there is sufficient moisture (Pérembelon 2002; De Boer 2003; Smadja et al. 2004), conditions which are not uncommon in greenhouses containing wasabi plants. The bacteria produce cell-wall degrading enzymes to induce soft rot on a range of host tissues (Barnard and Salmond 2007). Soft rot symptoms on wasabi crowns and petioles have been observed in a commercial greenhouse in which *P. carotovorum* was recovered during the warm summer months (authors, unpublished observations).

The potting mix used by commercial growers for vegetative propagation of wasabi was shown to contain *P. carotovorum* and is likely to provide a primary source of inoculum. Since vegetative offshoots are cut from the main rhizome using knives, this can provide entry wounds for direct vascular colonisation by bacteria, and is likely to also spread the pathogen. Besides the potting mix, vegetatively-propagated asymptomatic plants may harbour *P. carotovorum* (Goto and Matsumoto 1986). The recommendation of using only two successive generations of vegetatively-propagated plants has been reported to decrease the frequency of vascular blackening symptoms (Adachi 1987; Chadwick et al. 1993), presumably by reducing inoculum carry-over into successive generations.

Infection by *Pythium* species, which destroy lateral roots and the epidermis of the rhizome, can also enhance infection by *P. carotovorum* as shown in this study. In plantings initiated from seed, where vegetative offshoots are not used and wounds are minimised, the incidence of rhizome vascular blackening

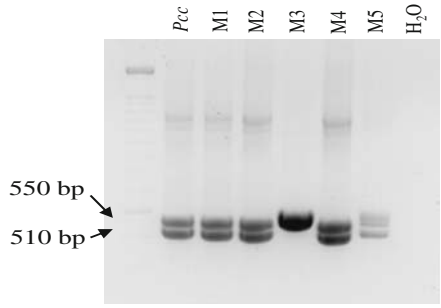


**Fig. 4** Identification of *Pcc* using primer set L1r and G1f. Fragment sizes were ~510 and ~550 bp. *Lanes M1* to *M5* represent isolates which exhibited pectolytic activity after dilution-plating of potting mix onto crystal violet pectate medium (CVP). *M1*, *M2* and *M4* exhibited the same banding pattern as *Pcc*, the positive control isolate originally obtained from symptomatic wasabi, while *M3* and *M5* showed pectolytic activity on CVP but were not *Pcc* as demonstrated by PCR. Water was included as a negative control ($H_2O$)

can be high if there is damage by *Pythium* species, which are prevalent in wasabi greenhouses in B.C. (Rodriguez and Punja 2007). Recent efforts to reduce *Pythium* infection by use of selective fungicides have shown a dramatic reduction in the development of internal rhizome blackening symptoms (authors, unpublished observations).

In summary, our study shows that *Pcc* is a likely cause of internal rhizome vascular blackening of wasabi, in which wounded roots or cut rhizomes provide entry points for the bacteria. The use of certified pathogen-free plants derived from meristem-tip culture may reduce rhizome blackening, provided there is no inoculum being introduced from the potting medium. Tissue-cultured plants would also not incur wounding when compared to vegetative propagation, thus reducing the potential for direct entry into the rhizome vascular tissue by *P. carotovorum*. In addition, management of root-infecting pathogens that provide avenues for entry of *P. carotovorum* has shown success in reducing vascular blackening.

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