INTRODUCTION

Enzymatic browning of cut surfaces is a problem with several fresh-cut products, since wounding of products stimulates phenolic metabolism and subsequently induces tissue browning (Watada and Qi, 1999). Phenylalanine ammonia lyase (PAL) is the first committed enzyme of primary phenolic biosynthesis (Saltveit, 2000), while oxidation of phenols catalyzed by polyphenol oxidase (PPO) results in browning complexes (Toivonen and Brummell, 2008). Peroxidase (POD) may also contribute to enzymatic browning in the presence of ongoing PPO-mediated browning reactions as a terminal enzyme involved in the polymerization and synthesis of lignin in apples (Valentines et al., 2005) and pears (Richard-Forget and Gauillard, 1997) that are rich in endogenous phenolics.

Enzymatic browning is endowed with defense response of plants to pathogenic infection and invasion. The mechanism of the disease resistance involves inducible defense responses in the form of defense-related enzymes such as PAL (Ngadze et al., 2012; Vanitha et al., 2009), PPO (Ngadze et al., 2012; Vanitha et al., 2009), and POD (Ngadze et al., 2012; Valentines et al., 2005) and the defense-related secondary compounds such as phenols (Barros and Saltveit, 2013; Ngadze et al., 2012; Vanitha et al., 2009) and lignin (Valentines et al., 2005). Accumulation of antimicrobial phenolic compounds in plant tissues can increase the tissues resistance to microbial growth (Barros and Saltveit, 2013), and lignification can enhance the mechanical strength of cell walls to deter microbial invasion (Valentines et al., 2005). On the
other hand, with respect to plant-pathogen interactions, plant growth promoting rhizobacteria (PGPR) including fluorescent pseudomonads, which are non-pathogenic rhizobacteria, are known to enhance plant resistance to soilborne pathogens through rhizosphere colonization, antibiotic, and inhibitory siderophore production (Janisiewicz and Korsten, 2003). In addition to the direct interaction with pathogens, PGPR can induce systemic resistance in plants by activating defense-related enzymes and secondary compounds (Ramamoorthy et al., 2002). Thus, inoculation with fluorescent pseudomonads may stimulate enzymatic browning of fresh-cut products and act as a biological control agent.

The browning reaction of fresh-cut potatoes can start immediately after wounding because potatoes contain many endogenous phenolic compounds that serve as a potential substrate for the PPO. Inoculation with certain fluorescent pseudomonads onto fresh-cut potatoes is proposed to induce expression of defense genes encoding PPO and increase PPO activity and phenolic compounds by induced systemic resistance (ISR) (Ramamoorthy et al., 2002). Enhanced activity of PPO in tomatoes by inoculation with specific fluorescent pseudomonads has also been demonstrated in relation to ISR (Ardebili, et al., 2011; Vanitha and Umesha, 2011). However, it is not clear whether the raised levels of PPO are derived from the plant or released by bacteria because PPO seems to be ubiquitous in plants and microorganisms. The type of PPO in microorganisms is probably different from that in plants, but there is some evidence indicating that both types of PPO have roles in defense against pathogens (Mayer, 2006). It will lead to infer the interactions of action of Pseudomonas species and enzymatic browning reactions to investigate which of the PPO is involved in browning response. Hence, the present study was conducted to (1) correlate fluorescent pseudomonads and browning severity on fresh-cut potatoes and (2) to establish the influence of inoculating Pseudomonas fluorescens isolated from the brown tissues of fresh-cut potatoes on synthesis of plant PPO and bacterial PPO during the browning response.

MATERIALS AND METHODS

Plant materials and storage Potatoes (Solanum tuberosum L. cv. Dansyaku) purchased from a local market in the Wakayama Prefecture were washed with tap water to remove soil adhering to the surface, and then peeled. About 700 g slices (3 cm × 3 cm × 0.5 cm thick) were prepared with knife. Ten slices were placed onto a plastic tray and put into unsealed LDPE film, and then the packages were stored overnight at 20°C or 3 days at 5°C to develop brown discoloration. Three replicated samples were prepared, and all analyses were carried out with three technical replicates for each treatment.

Evaluation of browning Ten slices in each package were evaluated for severity of browning. Samples were visually scored on a scale of 0 to 3, with 0 = no browning, 1 = slight browning, 2 = moderate browning, 3 = severe browning.

Bacterial counts A 10-g sample was macerated in 90 mL sterile saline solution (0.85 g/100 mL NaCl in water) in a sterile stomacher bag with an Elmex stomacher (Promedia SH-001; ELMEX, Tokyo, Japan) for 5 min at room temperature. Serial dilutions of this solution were made using sterile saline solution and then poured onto duplicate standard method agar (SMA; Nissui Pharmaceutical, Tokyo, Japan) plates. After incubation at 37°C for 48 h, bacterial counts were enumerated, and the bacterial plate count was expressed as log CFU/g of potatoes.

Bacterial isolation and identification The diluent of each sample was plated onto the surface of solidified SMA. Bacteria were aseptically isolated from the SMA plates incubated at 37°C for 48 h, and a total of 96 bacterial isolates were selected from different-appearing types of colonies growing on the petri plates from all samples. A MicroSeq microbial identification system (Applied Biosystems, California, USA) was used to identify bacteria. The sequencing data were analyzed using Analysis Software (MicroSeq analysis software Version 2.0, MicroSeq. 16S rDNA sequence databases Version 2.2) as previously described (Poubol and Izumi, 2005). A cutoff of the highest matching score with the sequence in the database was chosen for species identification.

Bacterial culture and inoculation Six isolates including P. fluorescens, Curtobacterium albidum, C. luteum, Leifsonia aquatica, Microbacterium oleovolans, and Citrobacter freundii were isolated only from the severely browning tissues of potato slices and used in artificial inoculation trials. Cells were grown in 25 mL standard method broth (SMB: 2.5 g yeast extract, 5.0 g peptone, and 1.0 g D(+)glucose in 1 L) for 16-20 h at 30°C and harvested by centrifugation for 10 min at 3000 x g at 20°C. The bacterial cells were washed twice in distilled water and adjusted to approximately 10^3 CFU/mL with distilled water for inoculation using nephelometry with a cell density meter reading at 600 nm (CO8000, Biochrom Ltd., Cambridge, UK). A 300 g sample of potato slices were dipped in 3 L of suspension of each bacteria for 30 min. Non-inoculated slices
dipped in distilled water were prepared as the control. After dipping, non-inoculated and inoculated samples were dried in a laminar flow hood for 30 min in preparation for the following browning potential analysis.

**Measurement of phenolic content** Phenolic content was measured by the method described by Kang and Saltveit (2002) with some modifications. A 5-g sample was homogenized in 100 mL of 80 % (v/v) ethanol, and the 25 mL of ethanol extract was evaporated and then resuspended in 25 mL of deionized water. An aliquot, 2.5 mL of the sample, was added to 2.5 mL of 1 N Folin-Ciocalteau reagent and the solution was kept for 3 min at room temperature. After adding 2.5 mL of 10 % (wt/vol) Na2CO3, the reaction mixture was incubated at 30°C for 60 min. Chlorogenic acid was used as a standard. The absorption of the developed color was measured at 700 nm with a spectrometer (UV-1600; Shimadzu, Kyoto, Japan). The total phenolic content was expressed as mg chlorogenic acid/kg based on fresh weight.

**Assays of the enzymes involved in enzymatic browning** PAL activity was measured as described by Hyodo (1976) with some modifications. A 3 g-sample was homogenized in 10 mL of 0.1 M borate buffer (pH 8.5) containing 1.4 mM 2-mercaptoethanol and 0.3 g of insoluble polyvinyl polypyrolidone (PVPP) on ice. The homogenate was filtered through Toyo No. 2 filter paper, and the solid fraction was centrifuged at 12000 x g for 30 min at 2°C. The supernatant was used for the PAL enzyme assay. The reaction mixture contained 1 mL of enzyme extract, 0.5 mL of 0.1 M borate buffer (pH 8.5), and 0.5 mL of 40 mM L-phenylalanine. The reaction mixture without L-phenylalanine served as the control. Reaction tubes were incubated for 1 h at 30°C and 0.5 mL of 2 M perchloric acid was added to stop the reaction. After 1 mL of enzyme extract was added to the control tubes, the absorbance at 280 nm of each sample was measured spectrophotometrically. PAL activity was expressed as change in absorbance at 280 nm/h/g fresh weight.

For determination of PPO and POD enzyme activity, a 5-g sample was homogenized in 10 mL of 10 mM sodium phosphate buffer (pH 6.0) and 0.5 g of PVPP on ice, centrifuged at 12000 x g for 20 min at 2°C, and the supernatant was used. For POD assay, the reaction mixture consisted of 50 μL enzyme extract, 200 μL of 10 mM L-β-(3,4-Dihydroxyphenyl) alanine (L-DOPA), and 750 μL of diluted Macilvain buffer (pH 4.5). POD activity was measured by increase in absorbance at 475 nm according to a modified method of Okumura et al. (2011) and determined as mg chlorogenic acid/min/g fresh weight.

The activity of POD was estimated using 100 μL of enzyme extract in 10 mM phosphate buffer (pH 6.0) containing 100 μL of 600 mM hydrogen peroxide as an oxidant and 7.5 μL of 100 mM guaiacol in 50 mM phosphate buffer (pH 6.0) as a hydrogen donor. The oxidation of guaiacol was measured at 30°C using a spectrophotometer reading at 420 nm. POD enzyme activity was defined as the increase in absorbance per min and expressed as change in 420 nm absorbance/min/g fresh weight (Vanitha and Umesha, 2011).

**Activity of plant PPO and bacterial PPO at different pH** For preparation of *P. fluorescens* cell extract, *P. fluorescens* was grown in 200 mL of SMB at 30°C for 3 days and centrifuged for 10 min at 5000 x g at 2°C. Cells were washed twice in phosphate buffered saline (pH 7.5) and resuspended in 4 mL of 10 mM phosphate buffer (pH 6). The bacterial suspension was sonicated using an ultrasonic homogenizer UX-050 (Mitsui Electric, Chiba, Japan) on ice and then centrifuged at 13000 x g for 15 min at 2°C. The supernatant was used for bacterial PPO assay. Activity of plant PPO derived from potatoes and bacterial PPO released by *P. fluorescens* were determined at pH range from 3.0 to 8.0 in diluted Macilvain buffer consisting of 50 mM phosphate and 25 mM citrate for PPO substrates such as monophenol tyrosine and di-phenol catechol and chlorogenic acid. The reaction mixture consisted of 50 μL of enzyme extracts, 200 μL of 40 mM substrate, and 750 μL of diluted Macilvain buffer. POD activity was measured by increase in absorbance at 410 nm per min. (Mizobutsi, et al., 2010).

**Analysis of plant PPO gene expression by real-time quantitative reverse transcription PCR (qRT-PCR)** Total RNA was extracted from potato tissues using an Isogen kit (Nippon gene, Tokyo, Japan) according to the manufacturer’s instructions. Purified total RNA samples were treated using the Ambion DNA-free kit (Life technologies, California, USA) to eliminate potential contamination from genomic DNA. First strand cDNA was synthesized from 0.5 μg of total RNA using reverse RTase and oligo (dT) primer (Takara, Shiga, Japan) for eukaryote RNA and amplified using MiniOpticon real-time PCR detection system with CFX Manager software (BioRad, California, USA) with the following primer pairs to match the strands of *PPO* genes (Ch i et al., 2014): *elongation factor-1 alpha* (Ef1α) forward (5’-ATTGGAAACGGATATGCTCC-3’) and reverse (5’-TCTTTACCAGGCAAGCTCTGCTC-3’), *PPO1* forward (5’-GACCAGCTTCGTCAAGGAC-3’) and reverse (5’-TTGTCGAAGCTTCCAGGACAC-3’), *PPO2* forward (5’-CGCGACTTGTGATTTCCA-3’) and reverse (5’-TGATCGTCGACCTCTC-3’), *PPO3* forward (5’-TTGTCGAAGCTTCCAGGACAC-3’), and reverse (5’-TGATCGTCGACCTCTC-3’).
forward (5'-TTACGCACCAATGCCAAC-3') and reverse (5'-CCATCTTCGTGAGTGGAAC-3'), PPO4 forward (5'-TCTGGTGCCAAAGAAAGTAA-3') and reverse (5'-CAAAATCCGCAGATTCAA-3'), and PPO9 forward (5'-TGAAAACGAAAAACGAAGC-3) and reverse (5'-CCGAACACCTTCTTGGTACA-3). A 20 μL mixture was comprised of 10 μL of SSofast EvaGreen Super Mix (BioRad), 5 μM of forward primer and 5 μM of reverse primer and 1 μL of cDNA. The thermal cycling was programmed as follows: 95°C for 30 s followed by 40 cycles at 95°C for 5 s and 60°C for 10 s. At the end of the qRT-PCR run, a melting curve analysis with temperature gradient from 65°C to 95°C at 0.1°C/s was also performed to ensure that only a single product was produced. Relative expression levels of the PPO genes were calculated based on a method described by Paffl (2001). Data normalization was performed using the gene expression values of EF1α in each sample.

**Statistical analysis** Experiments were conducted in a completely randomized design with a minimum of 3 replicates. Data were calculated as averages ± standard error and the significance of differences ($P \leq 0.05$) between and among samples within each point of analysis was determined for data based on t tests and analysis of variance (ANOVA), respectively.

**RESULTS**

**Bacterial flora of non-browning and browning tissues of potato slices** Bacterial counts of sliced potatoes increased from 2.6 log CFU/g in non-browning tissues (score 0) on the initial day to 3.7 log CFU/g in slightly browning tissues (score 1), 3.9 log CFU/g in middle browning tissues (score 2), and 4.6 log CFU/g in severely browning tissues (score 3) after 1 day of storage at 20°C (Fig. 1). Bacterial counts increased with increased severity of browning. A wide diversity of bacterial flora of potato slices was found in slightly browning tissues (29 species belonging to 19 genera) and severely browning tissues (31 species belonging to 16 genera) (data not shown). The commonly isolated genera from both tissues included gram-negative bacteria such as Enterobacter, Erwinia, Pantoea, and Burkholderia, belonging to phytopathogenic organisms, and gram-positive bacteria such as Arthrobacter and Bacillus, belonging to soilborne organisms. The exceptions were P. fluorescens, Curtobacterium albidadum, C. luteum, Leifsonia aquatica, Microbacterium oleivorans, and Citrobacter freundii, which were isolated only from the severely browning tissues. Among the six bacteria species, only inoculation with P. fluorescens developed browning discoloration on potato slices (data not shown).

**Browning reactions of potato slices either non-inoculated or inoculated with Pseudomonas fluorescens** When potato slices were initially inoculated with $10^5$ CFU/ml of P. fluorescens and then stored at 5°C, bacterial counts increased from an initial 2.9 log CFU/g to 5.4 log CFU/g after 3 days of storage (Fig. 2A). Inoculation with $10^3$ CFU/ml of P. fluorescens enhanced surface browning of slices (Fig. 2B) and increased the phenolic content about 20 % from its initial level (Fig. 2C) on day 3, while no browning and no accumulation of phenolics were found in non-inoculated slices during storage.

Potato slices had much lower PAL activity as compared to PPO and POD activities, but the activity was higher in slices inoculated with P. fluorescens on the first day after inoculation than non-inoculated controls (Fig. 3A). Thereafter, PAL activity in inoculated samples decreased by day 3 after inoculation as compared to the control samples. PPO activity started to increase 1 day after P. fluorescens inoculation and increased until 3 days of storage, when the activity was as much as twofold higher than the control (Fig. 3B). No differences were found in POD activity between non-inoculated and inoculated samples, and POD activity remained almost unchanged throughout 3 days of storage (Fig. 3C).

**Affinity of plant and bacterial PPO substrates at pH 3-8** We revealed that the extracts of P. fluorescens cultured at 30°C has PPO activity (0.17 Δ475 nm/ min/mg protein) (data not shown). Since pH generally influences an enzyme’s affinity for its substrate, activities of plant PPO and bacterial PPO were determined.
FIG. 2. Bacterial counts (A), browning score (B), and phenolic content (C) in potato slices either non-inoculated or inoculated with *P. fluorescens* during storage for 3 days at 5°C. Means with * indicate significance at $P \leq 0.05$ between non-inoculated and inoculated samples within the same day. Vertical lines represent the standard error (SE) of the mean ($n=3$). Browning score was rated on a scale of 0 to 3, with 0 = no browning, 1 = slight browning, 2 = moderate browning, 3 = severe browning.

FIG. 3. Activities of PAL (A), PPO (B), and POD (C) in potato slices either non-inoculated or inoculated with *P. fluorescens* during storage for 3 days at 5°C. Means with * indicate significance at $P \leq 0.05$ between non-inoculated and inoculated samples within the same day. Vertical lines represent the standard error (SE) of the mean ($n=3$).
in reactions ranging from pH 3 to 8 using the traditional PPO substrates catechol (Fig. 4A), chlorogenic acid (Fig. 4B), and tyrosine (Fig. 4C).

With catechol as the substrate, PPO activity in potato slices either non-inoculated or inoculated with *P. fluorescens* increased with increases in pH and reached a maximum at pH 7. Maximum PPO activity from *P. fluorescens* extracts was recorded at pH 5, and then activity drastically declined to an inactivation level at pH 6-8.

Similar to the pH-dependent affinity for catechol, maximum PPO activity with chlorogenic acid as the substrate was observed at pH 6 in both non-inoculated and inoculated potato slices and pH 5 in bacterial extracts. On the other hand, the activity of PPO with tyrosine as the substrate was maximal at pH 3-4 and declined drastically at pH 5 in extracts from both plant and bacteria. This result indicated that tyrosine had little affinity as the substrate for PPO activity in potato tissues at around pH 6.

Plant PPO gene expression in potato slices either non-inoculated or inoculated with *Pseudomonas fluorescens* Relative transcript levels of 5 genes coding for plant PPO contributing browning reactions were compared between potato slices non-inoculated and inoculated with *P. fluorescens* (Fig. 5). The expression of only the *PPO1* gene in slices inoculated with *P. fluorescens* was approximately two times higher than that in non-inoculated slices. There were no significant
differences in the transcript of other PPO genes (PPO2, PPO3, PPO4, and PPO9) between non-inoculated and inoculated samples. The variation (SE) in PPO9 gene expression between samples was large.

**DISCUSSION**

The wound-induced enzymatic browning has been shown to be related to bacterial growth on fresh-cut products and the browning is presumably a defense response of plants to control microbial growth by the synthesis of defense-related enzymes and secondary compounds (Ngadze et al., 2012; Valentins et al., 2005; Vanitha et al., 2009). With lettuce, heat treatment suppressed wound-induced accumulation of phenolics and increased susceptibility of the tissues to microbial growth (Barros and Saltveit, 2013), and bacterial inoculation following heat treatment showed no discoloration and induced bacterial growth on their surface (Rodov et al., 2015). These results support enzymatic browning as a way to prevent the tissues from becoming a hospitable host for microbial growth as a part of ISR. With potatoes in this study, we indicated that the two processes of enzymatic browning and microbial growth were interrelated because browning tissues had higher bacterial counts as browning intensified. Since the principal bacterial species isolated only from the severely browning tissues were identified as *P. fluorescens* that has been found to lead ISR (Ramamoorthy et al., 2002), we postulated that either synthesis of plant PPO derived from potatoes or bacterial PPO released by *P. fluorescens* would induce the browning reactions of fresh-cut potatoes.

The present study illustrated that inoculation with *P. fluorescens* induced PPO activity and accumulation of phenolic content in potatoes and encouraged their surface browning. Activities of PAL and POD were not stimulated by *P. fluorescens* treatment, although the POD enzyme in phenol-enriched produce could enable significant levels of POD-mediated polyphenol browning (Toivonen and Brummell, 2008). Thus, it remains unclear whether POD-mediated browning is consistently a significant component in potato browning. We observed PPO in both potatoes and *P. fluorescens*, and the plant PPO contributed to browning reactions rather than bacterial PPO because the plant PPO was activated, but bacterial PPO was suppressed with diphenol substrates such as catechol and chlorogenic acid in the pH range from 6 to 7 that is present in vegetable tissues.

It is conceivable that the browning process enhanced by endogenous phenolics in potatoes is mainly driven by the action of PPO based on genetic modification experiments. Coetzer et al. (2001) reported that the downregulation of PPO genes resulted in reduced PPO activity and reduced browning in the major commercial potato cultivar. Chi et al. (2014) revealed that 9 PPO-like gene models, named StuPPO1 to PPO9 (*Solanum tuberosum* PPO1 to 9), were discovered in potato tissues. *StuPPO1*, *PPO2*, *PPO3*, and *PPO4* genes were the major contributors to the total protein content, and each PPO gene contributed differently to browning. *StuPPO9* expression may be induced in response to the pathogen-infected potato tissues as disease defense and cell rescue (D’Ippolito et al., 2010; Tian et al., 2006). We also implicated that the PPO1 gene was expressed prominently in potato tissues following inoculation with *P. fluorescens*, in agreement with results of Ramamoorthy et al. (2002) on tomato roots treated with *P. fluorescens*. Li and Steffens (2002) demonstrated that transgenic tomato overexpressing a potato PPO cDNA showed strong inhibition of bacterial growth and exhibited a great increase in resistance to bacterial pathogens. Thiyapong et al. (2004) introduced antisense potato PPO cDNA into tomato plants, and the downregulation of PPO in antisense plants resulted in increased bacterial growth and enhanced susceptibility to bacterial pathogens.

Therefore, our results clearly demonstrated that enzymatic browning of fresh-cut potatoes involved phenolic oxidation through induction of plant PPO in plant defense reactions, where *P. fluorescens* could act as a strong inducer. Populations of *P. fluorescens* on fresh-cut potatoes could be affected by antagonism (Izumi, 2020) and quorum sensing (Juhas et al., 2005) between bacteria of same or different species or genera that present on potatoes. In regard to quorum sensing systems, the cell-to-cell communication systems involve in population density sensor, regulation of virulence, biofilm formation, and food spoilage, and are widespread in pseudomonads including *P. aeruginosa*, *P. putida*, *P. chloraphis*, and *P. fluorescens* (Ammor et al., 2008; Juhas, et al., 2005). Further research is needed to clarify the relationship between *P. fluorescens* and competitive bacteria on potatoes in a population density-dependent manner.

In conclusion, enzymatic browning was confirmed to be involved in a plant-bacteria interaction in fresh-cut potatoes and *P. fluorescens* in particular induced defense genes encoding plant PPO such as *PPO1*, plant PPO activity, and accumulation of phenolic compounds thereby enhancing browning in plant defense reactions. Plants have endogenous defense mechanism that can be induced in response to attack by pathogens, and *P. fluorescens* appears to systemically activate the plants’ latent defense mechanisms by serving as an inducer. Since these results also suggest that control of browning based on heat treatment and inhibitor treatment may result in aggravated microbial proliferation, the presence
of epiphytic and foodborne microorganisms must be taken into consideration to ensure microbial quality and safety when applying anti-browning strategies.

ACKNOWLEDGMENT

This work was supported by JSPS KAKENHI Grant Number 16K07606.

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