Effect of Lemon Balm Water Extract on Fusarium Wilt Control in Strawberry and Antifungal Properties of Secondary Metabolites

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In this study, the in vivo potential of lemon balm water extract on Fusarium wilt control in strawberry and the antifungal properties of secondary metabolites in the extract were investigated. Runner plants of strawberry (Fragaria × ananassa Duch., ‘Sachinoka’) were treated with water extracts (20%, w/v) of lemon balm (Melissa officinalis L.) and inoculated with Fusarium oxysporum f. sp. fragariae (Fof). Four weeks after Fof inoculation, lower disease incidence and indices in both shoots and roots were observed in lemon balm-treated plants. These effects could be attributed to reduced Fusarium populations due to the fungistasis and fungicidal effects induced by the extract in the rhizospheric soil. Consequently, dry weights of shoots and roots in the plants treated with lemon balm extracts were higher than those of the control. Based on the results of ultra performance liquid chromatography-tandem mass spectrometer (UPLC-MS/MS) analyses, rosmarinic acid was the metabolite with the highest concentration and was also the most stable metabolite in the water extract. In addition, the antifungal effect of rosmarinic acid against Fof was confirmed by in vitro tests. Therefore, water extracts of lemon balm could suppress Fusarium wilt in strawberry plants and rosmarinic acid was one of the key metabolites with antifungal properties present in the water extract.

Key Words: Fusarium oxysporum f. sp. fragariae, Lamiaceae, rosmarinic acid, UPLC-MS/MS.

Introduction

Lamiaceae herbs contain several phenolic compounds, terpenoids, and glucosides as secondary metabolites, with beneficial effects such as antimicrobial and antioxidant activities (Martino et al., 2009; Stanojevic et al., 2010; Weerakkody et al., 2011). The antimicrobial and preservative activities of the essential oils (EOs) in Lamiaceae herbs have been well documented, primarily for agri-foods (Teixeira et al., 2013; Gomes et al., 2014). In addition, the antioxidative and antifungal effects of the EOs on plant pathogens in vitro have been reported in a few studies (Isman, 2000; Quintanilla et al., 2002; Nazzaro et al., 2017). However, the antifungal effects and properties of Lamiaceae herbs on plant disease control remain unclear.

Fusarium wilt of strawberry (Fragaria × ananassa Duch.), caused by Fusarium oxysporum f. sp. fragariae (Fof), is one of the most common diseases in strawberry worldwide (Golzar et al., 2007; Arroyo et al., 2009; Koike and Gordon, 2015). Chemical control, crop rotation, non-pathogenic strain inoculation, and use of resistant cultivars are the most commonly employed strategies to manage Fusarium, a soil-borne disease (Koike and Gordon, 2015). Chemical control can overcome the pathogen, unless a new strain emerges. However, this approach is not eco-friendly and costly. Additionally, it is difficult to develop resistant cultivars because several traits, such as fruit productivity and quality of fruits, have to be considered during the development of successful resistant cultivars (Schaart et al., 2011).

Lemon balm (Melissa officinalis L.), which belongs to the family Lamiaceae, is an important medicinal herb that has been widely used in traditional medicine (Meftahizade et al., 2010). Furthermore, the EOs of lemon balm have also found applications in pharmacology, phytopathology, and food preservation (Abdellatif et al., 2014). Quintanilla et al. (2002) reported that the EOs of some herbs, such as thyme (Thymus vulgaris), oregano (Origanum vulgare), lemon balm (Melissa
officinalis L.), and peppermint (Mentha piperita), inhibited the growth of Phytophthora infestans in a plate assay in vitro. In addition, the EOs of lavender and rosemary suppressed the growth of Botrytis cinerea in vitro (Soylu et al., 2010). The volatile compounds in the EOs, which accumulate in closed environments under in vitro conditions, were responsible for the inhibitory activity against the fungi. Therefore, the use of these EOs in field conditions is impractical because they would diffuse away from the applied surface, resulting in a decrease in the effective concentration and enabling the disease-causing organism to resume growth (Letessier et al., 2001). In addition, many such extracts, particularly the EOs, have been reported to possess phytotoxic effects in crops following foliar application at high concentrations (Letessier et al., 2001). Conversely, the use of water extracts containing non-volatile secondary metabolites is a viable solution in terms of an environmentally-friendly disease control approach. Water extract preparation is a relatively easy and inexpensive process compared with that for preparing EOs. In addition, as the extracts are non-volatile, they can remain effective for longer periods than EOs. However, bioassays of such extracts through application in plants in vivo are required to investigate their potential use in practical settings as the antifungal effects observed in vitro often differ from those observed in vivo (Benner, 1993). This study was conducted to evaluate the effect of lemon balm water extract on Fusarium wilt control in strawberry, and to determine the antifungal properties of secondary metabolites present in the water extract using Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS).

Materials and Methods

Growing lemon balm and preparation of its water extract

Lemon balm seeds (Melissa officinalis L.) were sown in plastic containers (31.9 cm × 26.4 cm × 15.3 cm) containing commercial soil (Supermix A; Sakata Seed Corp, Japan) and grown in a greenhouse. Eight weeks after sowing, the plants were uprooted and the shoots cryopreserved using liquid nitrogen. Frozen samples were ground in distilled water using a mixer while maintaining the concentration of the herbal extract at 20% (w/v). The extract was filtered and the filtrate was used as herb extract solution.

Bioassay of herb extract for Fusarium wilt control in strawberry

Strawberry runner plants (Fragaria × ananassa Duch., ‘Sachinoka’) were grown in pots (10.5 cm in diameter, 0.5 L) with autoclaved commercial soil (SM-2; IBIKO CORPORATION, Japan) and fertilized using slow-releasing granular fertilizer (Long Total 70 day type; N:P:K = 13:9:11; JCAM AGRI. Co., Ltd., Japan). After six weeks, water extracts (20%, w/v) of lemon balm shoots were poured (50 mL/plant) onto the rhizospheric soil around strawberry plants. For plants under control treatment distilled water was used (50 mL/plant). Fusarium oxysporum f. sp. fragariae strain (2S) was cultivated on potato-dextrose agar medium and incubated in dark conditions at 25°C for two weeks to facilitate sporulation. The conidia were harvested in potato sucrose liquid media and incubated in dark conditions at 25°C for seven days. The conidial suspension was then sieved and the concentration was adjusted to 10⁶ conidia/mL. The conidial suspension was inoculated in the rhizospheric soil of each strawberry plant (50 mL/plant) immediately following lemon balm extract treatment and distilled water treatment for herb-treated and control plants, respectively. Ten plants per treatment with three replicates were grown in a greenhouse from June to July, 2018 at a 30/24 ± 4°C day/night temperature with 12–13-h photoperiods (750–1000 μmol·m⁻²·s) and 60–70% relative humidity (natural condition). Four weeks after Fof inoculation, 10 plants were selected from each treatment and the symptoms of Fusarium wilt were assessed according to Li et al. (2010) i.e. percentage of diseased shoots (compatible leaves and petioles, dark brown color) using five scales: 1, < 20%; 2, 20–40%; 3, 40–60%; 4, 60–80%; and 5, 80–100%, and using three scales for roots (reddish brown root lesions and transparent rotted roots): 1, part-diseased; 2, half-diseased; and 3, all-diseased. The disease index was calculated using the following formula:

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\text{Disease index} = \frac{\sum (\text{number of plants} \times \text{number for degree of symptom})}{\text{Total number of plants} \times \text{maximum degree of symptom} \times 100}
\]

Ten plants from each treatment were separted into shoots (compatible leaves and petioles) and roots (crown and roots) and dried using a constant temperature drier (ETTAS 600B) at 80°C for 2 days. Then, dry weights of shoots and roots were measured.

Four weeks after FoF inoculation, the rhizospheric soil was collected from 10 plants to analyse Fusarium populations. Each soil sample (1 g) was diluted to 10⁻³ with distilled water. Komada medium, which is selective for Fusarium oxysporum (Komada, 1975), was used. The inoculated media were incubated in dark conditions at 25°C for five days to determine the population numbers, and these were expressed as colony forming units (CFUs).

Analysis of lemon balm water extracts using UPLC-MS/MS

From the cryopreserved samples of five plants, 0.6 g of lemon balm shoots were pulverized in a mortar with liquid nitrogen to give a fine powder and mixed with
3 ml ultrapure water to prepare a sample extract solution (20%, w/v). The sample solution was then centrifuged (13,000 rpm, 4°C, 15 min) and the supernatant was filtered through a sterilizing filter (0.45 μm; ADVANTECH Co., Ltd., Japan). The sample was centrifuged (13,000 rpm, 4°C, 15 min) using Nanosep 10K (Pall corporation, Tokyo, Japan) to remove proteins in the extract.

The samples were analysed using UPLC-MS/MS (Waters Corporation, Milford, USA). A reversed-phase column (ACQUITY UPLC BEH C18, 1.7 μm, 2.1 × 100 mm; Waters Corporation, Milford, USA) with a thermostat at 25°C was used for the analysis. The mobile phases comprised 0.1% formic acid in water (A) and acetonitrile (B) at a flow rate of 0.4 mL/min. The gradient profile was as follows: 0–6 min, 95% A; 6–12 min, 75% A; 12–30 min, 65% A; 30–40 min, 50% A; 40–45 min, 5% A; 45–55 min, 5% A; and 55–60 min, 95% A. The mass spectrometer (Xevo Q ToF MS; Waters Corporation, Milford, USA) analysed the mass range of electrospray ionization in negative mode at 50–1000 m/z; MS/MS collision was performed at 30V. A mass chromatogram of the m/z value of each component in the extract was prepared from the results obtained using retention time.

To confirm the presence of rosmarinic acid, caffeic acid and luteolin in the water extract of lemon balm, comparisons of the retention time and collision fragments of the extracts were made with those of standard rosmarinic acid, luteolin and caffeic acid. The herb extract was loaded for LC analysis and three major peaks (selected according to peak size) were selected from the retention time graph and after confirmatory LC analysis, the pertaining m/z values were subjected to MS/MS fragmentation. The resulting fragment patterns were then compared with those of standard rosmarinic acid, luteolin and caffeic acid derived in a similar way based on the retention time. In this way, it was confirmed whether the compound in the extract was the expected chemical found in the MassBank database by cross referencing.

Evaluation of several identified chemicals for antifungal effect against Fof

Two milligrams of rosmarinic acid, luteolin, and caffeic acid (identified in the water extract of lemon balm shoots) were separately dissolved in 40 μl of ethanol, and 960 μl of distilled water was added to each of the three solutions. Fof, purely cultured in PDA medium, was mixed with Czapek-Dox liquid medium (Czapek, 1902; Dox, 1910) and incubated in a growth chamber (25°C, in dark conditions) for two weeks. In total, 10 mL of the prepared solutions were separately added to freshly prepared Czapek-Dox liquid medium, and for the control, distilled water was added. To factor out the effect of ethanol used for the preparation of solutions, a simple ethanol solution (ethanol: distilled water = 1:24, v/v) was also evaluated for comparison. The prepared Fof conidial suspension (10⁶ conidia/mL) was added to each of the Czapek-Dox liquid media containing the different solutions and incubated for five days in a growth chamber (25°C, in dark conditions). At the end of incubation, the numbers of conidia were counted using a hemocytometer. The averages were calculated from nine replicates and Fof populations in the liquid media were enumerated and expressed as CFU/mL using the following formula:

Fof population per ml of liquid medium = average number of conidia in four corner cells × 10⁴

Statistical analysis

Mean values were analysed by students t-test for dry weights, disease index, colony forming units, and by Tukey’s multiple range test for the antifungal effects of rosmarinic acid, caffeic acid and luteolin at P < 0.05. All the analyses were conducted using XLSTAT 2012 pro statistical analysis software (Addinsoft, New York).

Results

Four weeks after Fof inoculation, the dry weights of shoots and roots in strawberry plants under control treatment were significantly lower than those treated with lemon balm extract (Fig. 1). In the control plants, the incidence of Fusarium wilt in shoots reached 100%, with 50% of plants scored as severity level 5 (Fig. 2A). As a result, disease incidence and severity of symptoms in the shoots of control plants were higher and worse, respectively, than those in lemon balm-treated plants. In the roots, disease incidence in the control plants also reached 100%, with 25% of the plants exhibiting the
all-diseased condition (Fig. 2A). Conversely, roots of lemon balm-treated plants exhibited lower disease incidence and severity of symptoms than the control plants; no roots exhibited the all-diseased condition under lemon balm treatment. The disease indices also significantly decreased in lemon balm-treated plants compared with the control in both shoots (32.5 vs. 82.5) and roots (37.5 vs. 62.5) (Fig. 2B).

Application of lemon balm extracts seemed to have a considerable suppressive effect on the total CFUs of Fof in the rhizospheric soil of strawberry plants (Fig. 3). CFUs in the soil of plants treated with lemon balm extract were below $5 \times 10^4$, while in the control they were as high as $26 \times 10^4$.

The analysis of lemon balm water extract was conducted by liquid chromatography-mass spectrometry (LC-MS) and is represented in the form of a chromatogram and spectrum graph (Fig. 4A, B). From the chromatogram, the most promising regions of compounds were observed with retention times ranging from 9.12 min to 15.70 min. The highest peak size was observed at 13.98 min, followed by 10.9 min and 9.93 min. From the spectrum graph, the presence of pseudo molecular ions $[M+H]$ at m/z of approximately 359, 295, and 179 was observed at the corresponding retention times. Cross-referencing the values in MassBank (https://massbank.eu/MassBank/) revealed that the compounds were rosmarinic acid, luteolin, and caffeic acid, respectively.

To confirm the presence of rosmarinic acid, luteolin, and caffeic acid, the chromatogram and spectrum of lemon balm water extract were compared with those of standard rosmarinic acid, luteolin, and caffeic acid (Wako Pure Chemicals Industries, Ltd., Japan) samples. The chromatogram and mass spectrum of rosmarinic acid are presented and explained as representative results of this study. The chromatogram of the lemon balm extract in confirmatory analysis showed the highest peak at 13.92 min, and this was selected for MS/MS collision fragmentation (Fig. 4C I, II). The pseudo molecular ion $[M+H]$ at m/z 359 pertaining to the retention time broke into characteristic collision fragment patterns at m/z 197, 179, and 161, and these were also found in the standard rosmarinic acid solution (Fig. 4D, F) at a similar retention time (Fig. 4E I, II). Therefore, the presence of rosmarinic acid was confirmed. The presence of luteolin and caffeic acid in the herb water extract was confirmed in a similar manner.

Evaluation of rosmarinic acid, luteolin, and caffeic acid for antifungal effects against Fof in vitro showed
promising results (Fig. 5). The Fof populations in the media containing rosmarinic acid, luteolin, and caffeic acid were considerably lower ($66 \times 10^4$, $51.3 \times 10^4$, and $65.7 \times 10^4$ CFU/mL, respectively) than those in the control ($131 \times 10^4$ CFU/mL). In addition, the ethanol solution used to estimate the effect of ethanol on fungal populations exhibited no significant differences compared with that of the control.

**Discussion**

Lemon balm (*Melissa officinalis* L.), belonging to the family *Lamiaceae*, is an important medicinal herb that has been widely used in traditional medicine (Meftahizade et al., 2010). It has also found various applications in pharmacology, phytopathology, and food preservation (Abdellatif et al., 2014). However, such activities are attributed to the volatile EOs present in the lemon balm (Sharafzadeh et al., 2007; Adinee et al., 2008). In addition, most reports documenting the activities were obtained from *in vitro* studies. *In vitro* studies are critical in the identification of plant extracts with potential agricultural applications, although *in vivo* evidence is required for their adoption for commercial use (Gorris and Smid, 1995). In this study, disease incidence in both shoots and roots of strawberry plants treated with lemon balm was considerably lower than in controls. In addition, a suppressive effect on Fusarium populations was observed in the rhizospheric soil, indicating the fungistatic effect of the lemon balm extract on the pathogen. Based on these findings, it can be stated that the secondary metabolites present in the water extract of lemon balm shoots have the potential to sup-

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*Fig. 4. UPLC-MS analysis of lemon balm water extract and identification of rosmarinic acid in lemon balm water extract by chromatogram and MS spectrum of the collision fragments using LC-MS/MS. A, chromatogram of lemon balm extract; B, spectrum of lemon balm extract; C (I) selected retention time of the extract; (II) LC data of the herb extract; D, MS/MS collision spectrum at 13.92 min; E (I) selected retention time of rosmarinic acid solution; (II) LC data of standard rosmarinic acid; F, MS/MS collision spectrum at 13.86 min.*
press Fusarium wilt in strawberry plants. In addition, the disease suppression led to better growth of strawberry plants, as evidenced by the increased dry weight of both shoots and roots.

The analysis of lemon balm water extracts using LC-MS represented as a chromatogram yielded several peaks at different retention times. The most critical regions of the secondary metabolites were observed with retention times ranging from 9.12 to 15.70 min. The three major substances within the identified retention time range had m/z values of approximately 359, 295, and 179 respectively. A comparison of the m/z values using MassBank revealed that the compound with the highest contents was rosmarinic acid and that the other two were luteolin and caffeic acid. To confirm the presence of rosmarinic acid in the extract, an LC-MS/MS analysis was conducted using standard rosmarinic acid. The presence of luteolin and caffeic acid was also confirmed through analysis similar to that for rosmarinic acid, although their concentrations and order among the constituents significantly varied in the supplemental experiments. Flavonoids like luteolin are reportedly less stable and have high concentration.

Regarding cell surface damage due to pilferage, it has been hypothesized that phenolic acids such as rosmarinic acid play a critical role as phytoanticipins in plants (Dixon, 2001). Bais et al. (2002) reported that the antifungal activities of rosmarinic acid were exerted through the breakage of intersepta in the mycelia of fungi. Such specific activity of rosmarinic acid against microorganisms makes it a potent and novel antimicrobial agent. The results of the current study further confirm the antifungal potential of rosmarinic acid. In our study, the fungal populations treated with rosmarinic acid were considerably lower than those in the control in vitro. The result is consistent with the decreased fungal populations in the rhizospheric soil of the strawberry plants in the bioassay in the current study. Therefore, the presence of rosmarinic acid in the water extract of lemon balm plays a key role in exerting the characteristict antifungal effects described in the text. Conversely, the methanolic/ethanolic extracts and EOs of herbs run the risk of rapid evaporation from the surfaces on which they are applied, potentially reducing the effective concentration of the active compound and enabling the disease-causing organism to resume growth (Letessier et al., 2001). However, in the current study, the antifungal effect of the water extract was observed up to one month after application as demonstrated by the decrease in Fof populations in the rhizospheric soil.

The procedure of extract preparation in this study was simple, inexpensive, and sustainable, and the concentrations of the extracts were comparable with those obtained using other extraction methods.

In this study, direct effects of the antifungal properties of lemon balm extract on Fof and subsequent disease suppression were observed. However, some indirect defense mechanisms such as induced systemic resistance could also play a key role in the development of disease resistance in crops through the accumulation of phytoalexins (Dalisay and Kuc, 1995; Colpas et al., 2009) and the higher antioxidant activity in the presence of herb extracts. Further analyses to confirm these possibilities are also required to determine the mechanism underlying disease suppression in herb-treated plants. In addition, other herbs in the Lamiaceae family should be screened for similar properties and to determine if they have similar suppressive effects on Fof. Furthermore, evaluation of their antifungal properties against other soil-borne diseases, including other crops and top part diseases like anthracnose, should be considered in future studies.

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