Metabolome Analysis of Lamiaceae Herbs and Growth Improvement in Vegetable Crops

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（シソ科ハーブのメタボローム解析並びに数種野菜における生育改善）

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CHAPTER 1

Antioxidative ability of several *Lamiaceae* herbs and evaluation of *in vitro* antifungal properties

Introduction

Plants have been the primary food source for humans since the beginning of time and will continue to be so. Besides being the means of sustenance, plants have also been utilized for overcoming various other obstacles that hurdled the progress of human race. One of such hurdles are the diseases that plagued humans both directly and indirectly. Plants have been the source for medicinal treatments for both as curative and preventive measures for thousands of years (Carović-Stanko et al., 2016). According to the World Health Organization (WHO), about 80% of the world population still relies mainly on plant-based drugs (Bahmani et al., 2014), thus lowering at the same time the impact of self-medication side effects (Alexa et al., 2014). Plants contains a vast array of natural compounds that are responsible for these actions which pave ways to novel remedies with less side effects compared to synthetics. Among the various plants used for this purpose, the *Lamiaceae* is one of the most important herbal families that has been used in folk medicine since ancient times. It contains about 236 genera and more than 6000 species and the largest genera are *Salvia* (900), *Scutellaria* (360), *Stachys* (300), *Plectranthus* (300), *Hyptis* (280), *Teucrium* (250), *Vitex* (250), *Thymus* (220) and *Nepeta* (200) (Raja, 2012). The most popular members of the family are thyme, mint, oregano, basil, sage, savory, rosemary, hyssop, lemon balm etc due to their aroma and flavor. They grow in different agroclimatic range and many of these are cultivated for use in cosmetics, flavoring, fragrance, perfumery, pesticides and pharmaceuticals industries (Özkan, 2008).

During normal metabolic functions in living organisms, several reactive compounds get produced in the form of reactive oxygen species (ROS). Beside the metabolic processes, environmental factors such as toxicants also serves as an elicitor of these reactive molecules like superoxide anion and hydroxyl radicals, hydrogen peroxide and singlet oxygen (Krishnamurty and Wadhwani, 2012). The presence of lone pair of electrons in their structure is the source of reactivity of these compounds which enables them to interact with other cellular

molecules rapidly. Generally, these compounds remain securely coupled to their site of generation and are eliminated by the endogenous antioxidative defense that ensures optimal cell function (Dastmalchi et al., 2007).

Plants generally being incapable of movement and lacking an immune system like animals, are prey to different external sources like herbivores and microbes. To protect against these external factors, plants have developed their own security system through production of several secondary metabolites in the evolutionary process. One of such defense mechanisms is the production of ROS. Upon pathogen invasion, high concentration of reactive oxygen species (ROS) gets produced in plants as a defense mechanism, a phenomenon known as oxidative burst. The concentration is usually higher than normal which could prove toxic to the invading pathogen. However, this mechanism is a double-edged blade as the excessive production of ROS could possibly overwhelm the plant's own antioxidant defense (Vanacker et al. 1998). This change in the oxidative balance, known as oxidative stress can result in degradation of cellular components viz. DNA, carbohydrates, polyunsaturated lipids and proteins, or precipitate enzyme inactivation, irreversible cellular dysfunction and ultimately cell death if the pro-oxidant-antioxidant balance is not restored (Dastmalchi et al., 2007). As such, the suppression of these ROS is important to ensure the proper growth of plants. Application of antioxidants exogenously can be a way to mitigate these excess ROS produced in plants that they cannot tackle endogenously. Reports about application of synthetic antioxidants to tackle this ROS production and inducing disease resistance are present (Gala and Abdou 1996; El-Gamal et al. 2007). However, in the wake of detrimental impact associated with synthetic chemical compounds, scientists are giving more importance to natural sources of such antioxidants. Many herbs especially from the family *Lamiaceae* are an excellent source of such natural antioxidants due to the presence of phenolic compounds in them. However, emphasis was given more on the aromatic or essential oils (EOs) and the related extraction process

(Triantaphyllou et al., 2001). As such, information regarding potential use of water extracts of *Lamiaceae* herbs as natural antioxidant resources in this aspect is scarce.

Beside the antioxidative effects of *Lamiaceae* herbs, scientists have delved in to the direct antifungal properties of the herb extracts against disease causing organisms in plants. Here also the emphasis was given more in to the EOs found in the extracts and their activity *in vitro* (Gomes et al., 2014). Although, the *in vitro* evaluation of such EOs had shown considerable success, transitioning these effects to field production system was difficult due to their innate volatile characteristics (Letesseir et al., 2001). Furthermore, phytotoxic effects were also observed in crops with excess application of such EOs. As such, a possible remedy to obtain the beneficial impact of the *Lamiaceae* herbs in disease control aspect is through the use of water extracts. Besides being nonvolatile, the water extracts of the herbs may not contain the harmful residues found in organic solvents (Hinneburg et al., 2006). However, information regarding the antifungal activity of *Lamiaceae* herbs water extracts *in vitro* is also scarce.

Therefore, the present study was conducted to evaluate the antioxidative activity of the water extracts of *Lamiaceae* herbs and subsequent *in vitro* antifungal activity against several Fusarium species. The findings would be used as a baseline for selecting potentially important herbs for further use.

Materials and Methods

Growing of *Lamiaceae* herbs: Seeds of 10 species of *Lamiaceae* herbs (Fig. 1), oregano (*Origanum vulgare* L.), catnip (*Nepeta cataria* L.), sage (*Salvia officinalis* L.), dark opal (*Ocimum* spp.), thyme (*Thymus vulgaris* L.), basil (*Ocimum basilicum* L.), hyssop (*Hyssopus officinalis* L.), peppermint (*Mentha piperita* L.), lamb's ear (*Stachys byzantina* K.) and lemon balm (*Melissa officinalis* L.), were sown in plastic containers (31.9 cm × 26.4 cm × 15.3 cm) containing autoclaved commercial soil (Supermix A, Sakata Co. Ltd., Japan) and grown in a greenhouse at 30 ± 4/24 ± 4 °C temperature with 12-13 h photoperiods (750-1000 µmol/m2/s)

and 60-70% relative humidity. Eight weeks after sowing, the plants were uprooted and the shoots and roots were cryopreserved using liquid nitrogen.

Measurement of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability: The DPPH radical scavenging ability was measured according to the method of Burtis and Bucar (2000) (Fig. 2). Frozen sample (0.1 g) was extracted using 3 ml of 80% ethanol and the extract was centrifuged at 13000 rpm, 4°C, 10 min. Then the supernatant (0.15 ml) was mixed with 0.9 ml of 400 μM DPPH solution, 0.9 ml of 0.2 M MES buffer solution (pH 6.0), 0.9 ml of 20% ethanol and 0.75 ml of 80% ethanol. Then the mixture was allowed to stand at room temperature for 30 min in dark condition. At that time, a blank was prepared by adding 0.15 ml of 80% ethanol. After completion of the reaction, the absorbance at 520 nm was measured with a spectrophotometer. Trolox (10 to 100 μg/ml) was used to create a calibration curve, and the blank measurement value was subtracted from the measurement value of the analysis sample, and the value calculated on the calibration curve was taken as the DPPH radical scavenging ability.

Polyphenol content determination: The method of MacDonald et al. (2001) using Folin-Denis reagent was adopted for the determination of the polyphenol contents in the prepared extracts, and absorbance was measured at 700 nm (Fig. 3). Frozen sample (0.1 g) was extracted by using 4 ml of 80% methanol and centrifuged at 13,000 rpm, 4°C, 10 min. Then the supernatant (0.2 ml) was mixed with 2.4 ml of distilled water, 0.2 ml of a Follin Denis reagent [distilled water 70 ml, sodium tungstate dihydrate 10 g, phosphomolybdic acid [12 molar (IV) phosphoric acid n-hydrate 2 g], 5 ml phosphoric acid and 0.4 ml saturated sodium carbonate. Then the mixture was allowed to stand in dark condition at 30ºC for 30 minutes. At that time, a blank was prepared by adding 0.2 ml of distilled water instead of Folin-Denis reagent. After completion of the reaction, the absorbance of the reaction mixture was measured at a wavelength of 700 nm using a spectrophotometer. Quercetin (1 to100 μg/ml) was used for the

calibration curve preparation, and the blank value was subtracted from the measurement value of the sample solution, and the value calculated from the calibration curve was taken as the polyphenol content.

Ascorbic acid content: For ascorbic acid, samples were extracted using 5% metaphosphoric acid at a ratio of 0.15 g/5 ml and analyzed as described by Mukherjee and Choudhuri (1983) (Fig. 4). Then the supernatant (0.5 ml) was mixed with 0.5 ml of 0.03% DCIP (sodium 2,6-dichloroindophenol solution), 0.5 ml of a 2% thiourea-5% metaphosphoric acid solution and 0.25 ml of 2% DNP (2,4-dinitrophenylhydrazine) solution. Then the mixture was kept in water bath at 50ºC for 70 min. After completion of the reaction, 2.0 ml of 85% sulfuric acid was slowly added while cooling in ice and the mixture was allowed to stand in dark condition at room temperature for 30 minutes. At the same time, the test tube which was used as blank (2% DNP solution) was also kept in dark condition but without adding the sulfuric acid solution. Then the absorbance of the reaction mixture was measured at a wavelength of 520 nm using a spectrophotometer. For the calibration curve creation, L-ascorbic acid (1 to100 µg/ml) was used, and the value obtained by subtracting the blank value from the measurement value of the sample solution was applied to the calibration curve to calculate the ascorbic acid content.

***In vitro* antifungal assay of the herb extracts:** Eight weeks after germination and before cryopreservation, ten plants were sampled in each herb and shoots and roots extracts (5 mg/ml and 20 mg/ml, w/v) were prepared by grounding in distilled water. The Fusarium species used for the *in vitro* assay were as follows:

1. *Fusarium oxysporum* f. sp. *fragariae* (Fof, 2S)
2. *Fusarium oxysporum* f. sp. *asparagi* (Foa, MAFF305556)
3. *Fusarium oxysporum* f. sp. *cyclaminis* (Foc, MAFF 712100)
4. *Fusarium oxysporum* f. sp. *lycopersici* (Fol, MAFF238900)
5. *Fusarium oxysporum* f. sp. *melonis* (Fom, MAFF242352)

The isolates grown on potato-dextrose-agar (PDA) were sub-cultured at 25 °C for 2 weeks in Czapek-Dox media (Czapek 1902; Dox 1910) containing $NaNO_3$ 3 g, K_2HPO_4 1 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, sucrose 30 g and agar 8 g/l (pH 5.8) (Fig. 5). The conidia were further subcultured (10^6 conidia/ml) in liquid Czapek-Dox media with or without addition of filter-sterilized water extracts (5 mg/ml and 20 mg/ml, w/v) of shoots and roots in each herb separately at 25 °C in the dark for few days by shaking the culture (100 rpm). Then, the density of conidia was investigated using hemocytometer, and the propagation index of extract-added plots to non-added plots was calculated using the following formula:

$$\text{Index of Fusarium propagation} = \frac{\text{Number of conidia in herb extract added plot}}{\text{Number of conidia in control plot}} \times 100$$

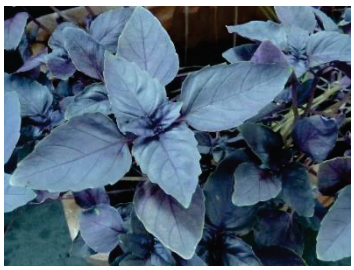
The average was calculated from 3 replications.

Statistical analysis: Mean values were analyzed by Tukey's multiple range test for DPPH radical scavenging activity, ascorbic acid content, polyphenol content and *in vitro* antifungal assay of the herb extracts at $P < 0.05$. All the analyses were conducted using XLSTAT pro statistical analysis software (Addinsoft, New York).

Oregano
Origanum vulgare L.

Catnip
Nepeta cataria L.

Sage
Salvia officinalis L.



Dark opal
Ocimum spp.

Thyme
Thymus vulgaris L.

Basil
Ocimum basilicum L.

Hyssop
Hyssopus officinalis L.

Peppermint
Mentha piperita L.

Lamb's ear
Stachys byzantine K.

Lemon balm
Melissa officinalis L.

Fig. 1. *Lamiaceae* herbs species used in the experiment.

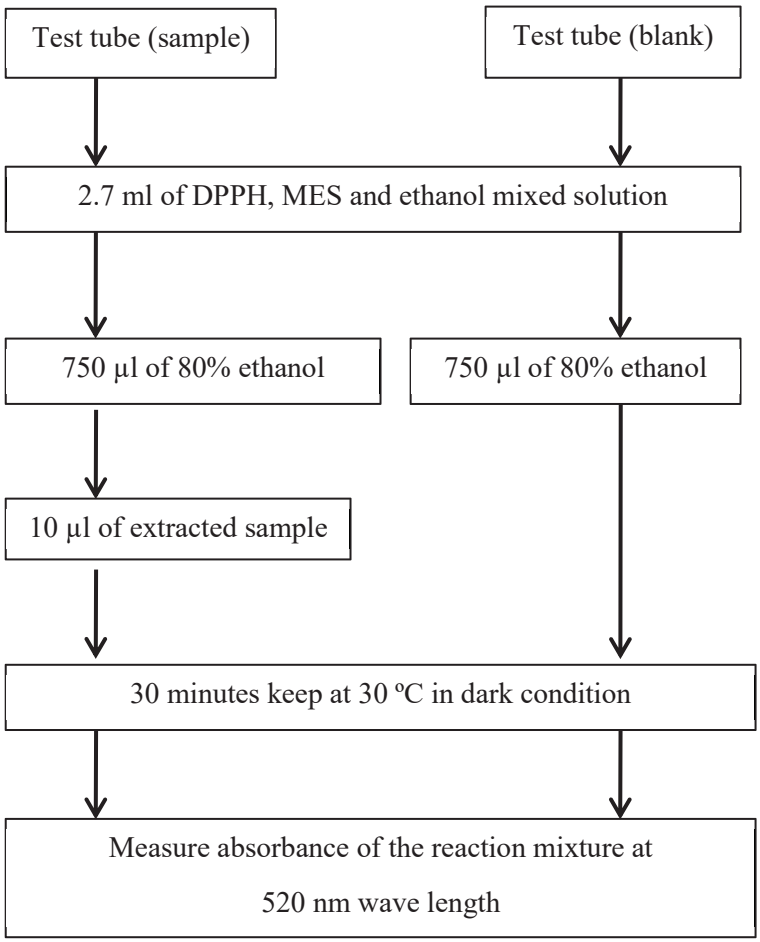


Fig. 2. Flow diagram of the procedures in DPPH radical scavenging activity analysis.

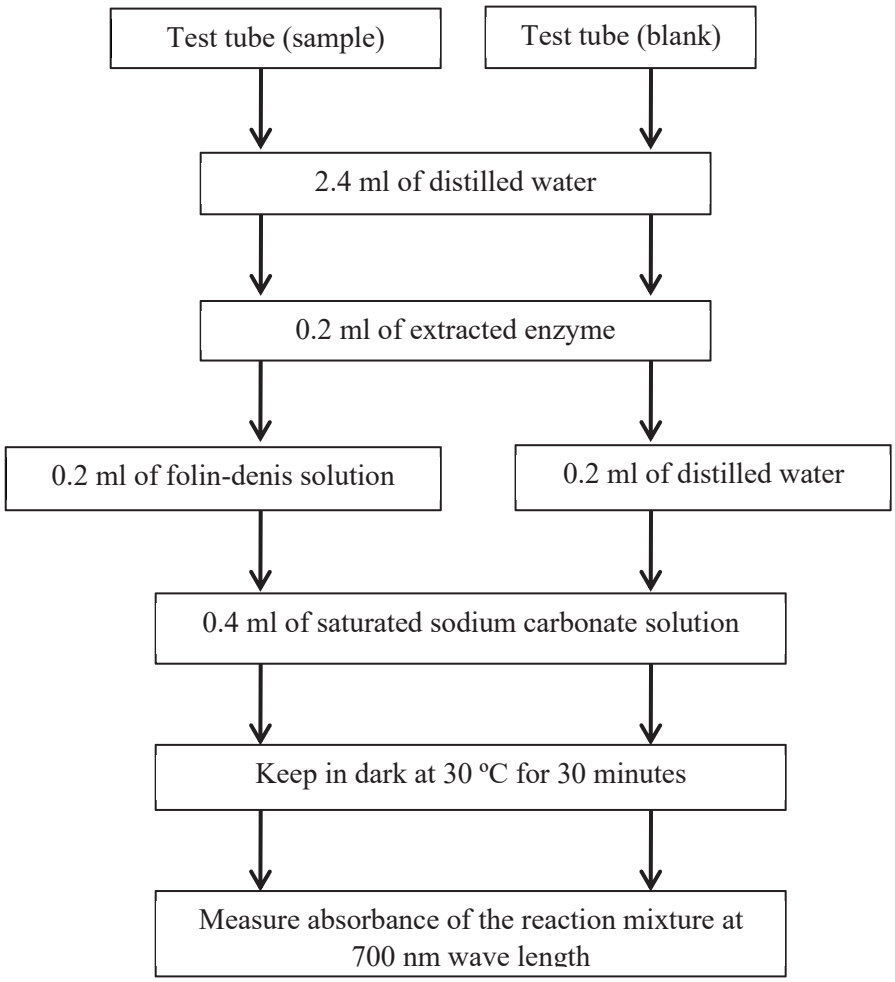


Fig. 3. Flow diagram of the procedures in polyphenol content assay.

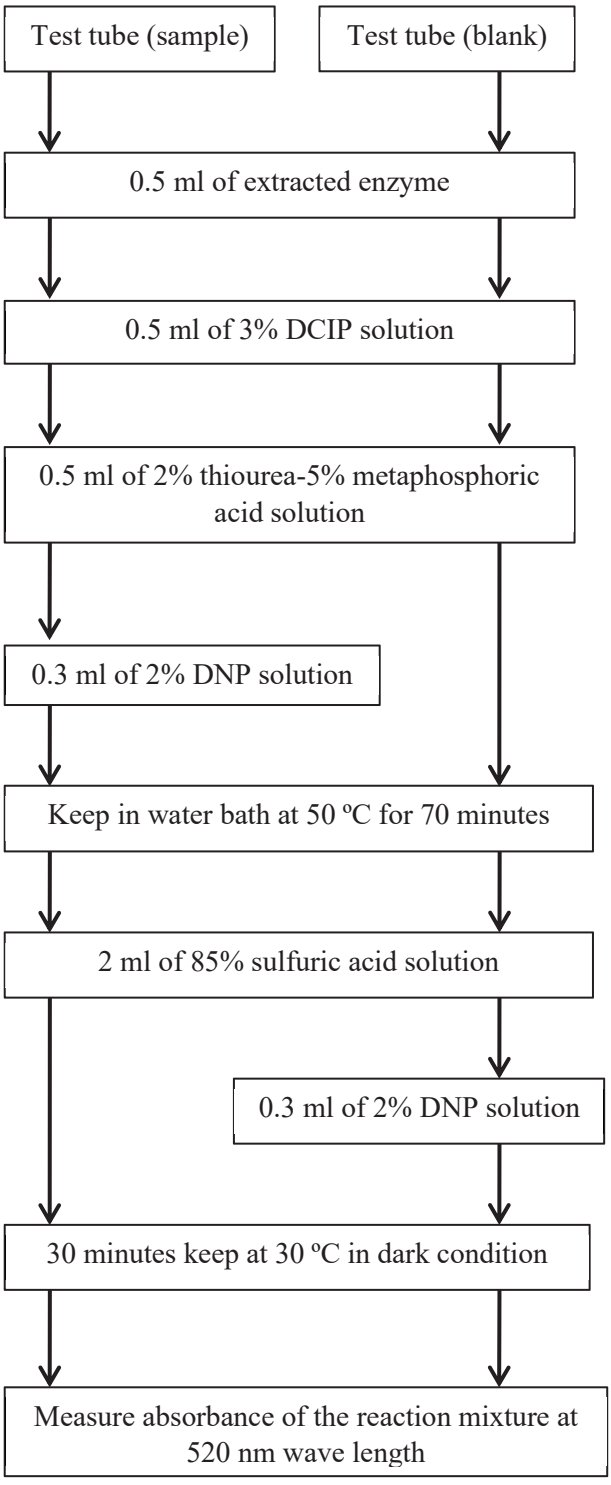


Fig. 4. Flow diagram of the procedures in ascorbic acid assay.

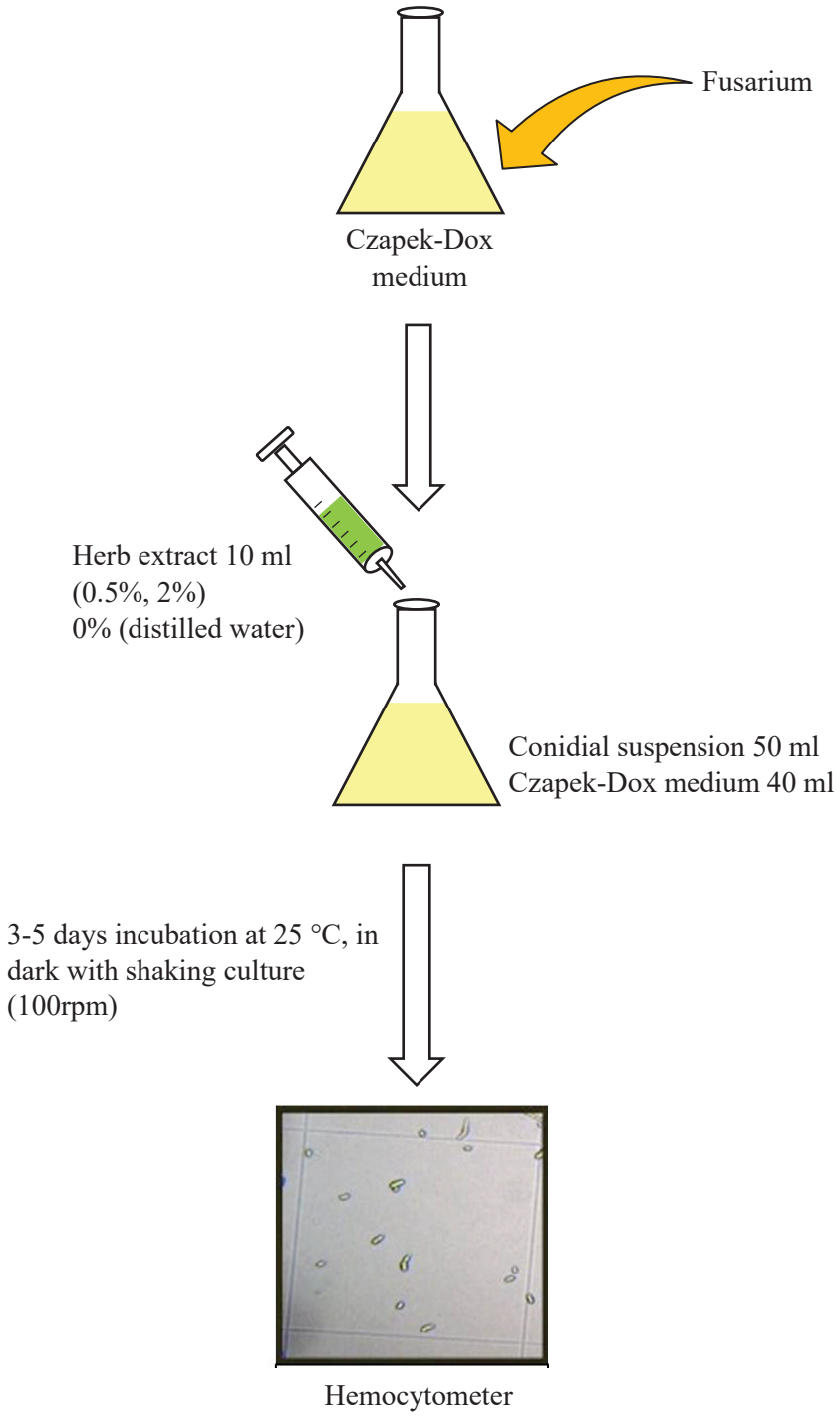


Fig. 5. *In vitro* antifungal assay of *Lamiaceae* herbs extracts.

Results

DPPH radical scavenging activity: The herbs evaluated in the experiment showed a varying degree of DPPH radical scavenging activity in both shoots and roots extracts (Fig. 6). Among the ten herbs, oregano, sage, hyssop and lemon balm showed the most radical scavenging activity in both shoots and roots extracts. In case of shoot extracts, among these 4 herbs, the highest activity was expressed by sage followed by oregano and hyssop with the lowest in lemon balm. Regarding root extracts, the highest radical scavenging activity was shown by hyssop among the 4 and the lowest was observed in lemon balm.

Polyphenol contents: Regarding shoot polyphenol contents (Fig. 7), highest was observed in oregano followed by sage, thyme and hyssop whereas the lowest was observed in lamb's ear. In case of root polyphenol content, highest content was observed in hyssop, to which basil and thyme showed statistical similarity. Catnip and oregano expressed the second highest content whereas the lowest was observed in peppermint to which lemon balm showed statistical similarity.

Ascorbic acid content: Regarding shoot ascorbic acid content (Fig. 8), the highest was observed in lemon balm. Among the rest, peppermint, catnip, oregano, sage and hyssop showed intermediate content whereas the lowest was observed in lamb's ear. On the other hand, in roots, the maximum ascorbic acid content was observed in oregano, followed by the other herbs with lowest observed in lamb's ear.

Antifungal activity of the *Lamiaceae* herbs extracts: In antifungal activity of the herb extracts *in vitro*, the extracts expressing a suppression of disease index below 100 were considered to be effective (Fig. 9-13). Regarding the two concentrations used, 2% extract in both shoots and roots of the herbs showed higher suppression rate in most of the Fusarium species considered compared to 0.5%. Among the 10 herbs evaluated, oregano, sage, hyssop

and lemon balm expressed the highest suppression in most of the Fusarium species except hyssop in Foa.

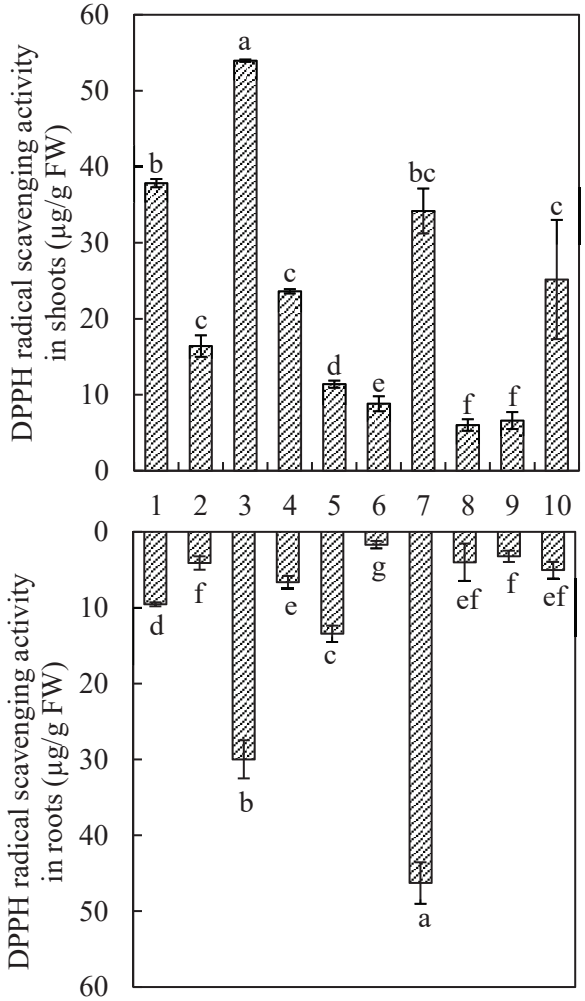


Fig. 6. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity in herbs. Here, 1, Oregano; 2, Catnip; 3, Sage; 4, Dark opal; 5, Thyme; 6, Basil; 7, Hyssop; 8, Peppermint; 9, Lamb's ear; 10, Lemon balm. Different letters represent significant differences among treatments by Tukey's test ($P < 0.05$).

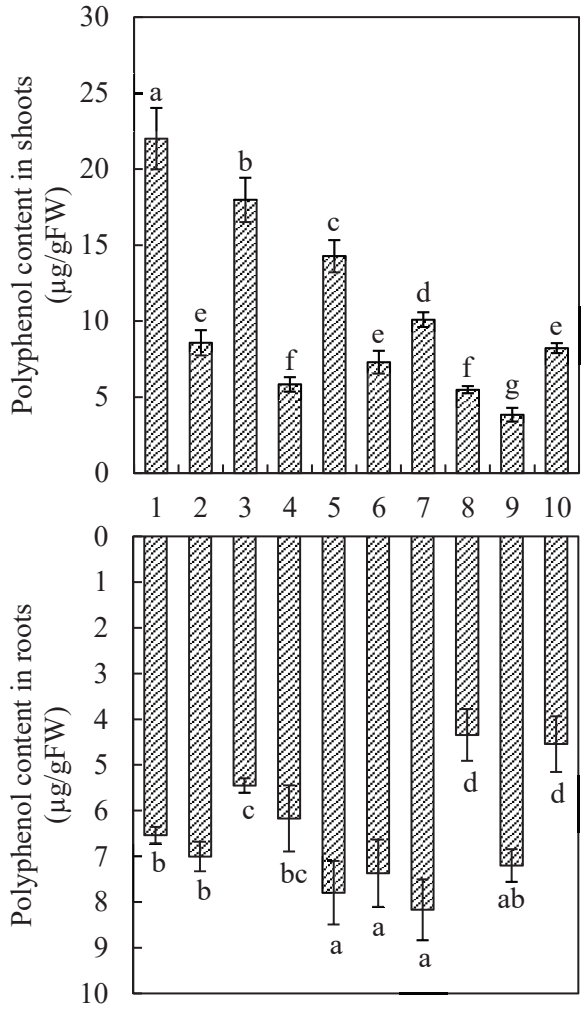


Fig. 7. Total polyphenol content in shoot and roots of herbs. Here, 1, Oregano; 2, Catnip; 3, Sage; 4, Dark opal; 5, Thyme; 6, Basil; 7, Hyssop; 8, Peppermint; 9, Lamb's ear; 10, Lemon balm. Different letters represent significant differences among treatments by Tukey's test ($P < 0.05$).

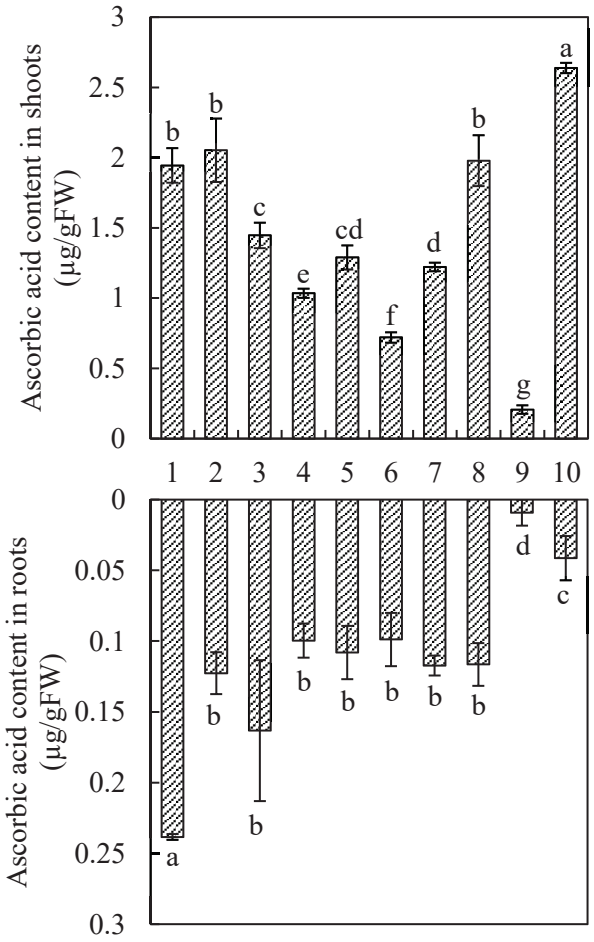


Fig. 8. Total ascorbic acid content in shoots and roots of herbs. Here, 1, Oregano; 2, Catnip; 3, Sage; 4, Dark opal; 5, Thyme; 6, Basil; 7, Hyssop; 8, Peppermint; 9, Lamb's ear; 10, Lemon balm. Different letters represent significant differences among treatments by Tukey's test ($P < 0.05$).

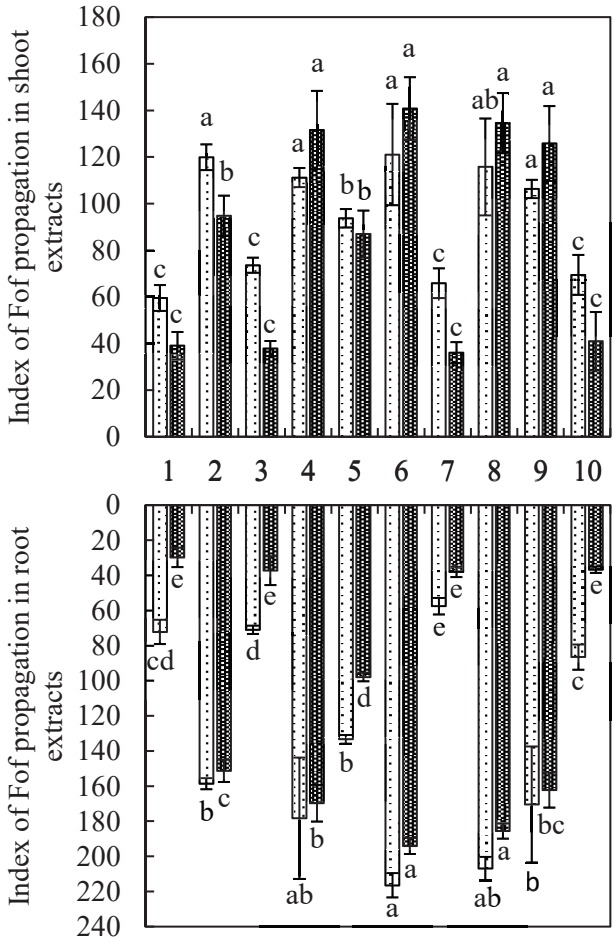


Fig. 9. Effect of herb extract on propagation of Fof (*Fusarium oxysporum* f. sp. *fragariae,* Fusarium wilt：2S). Here, 1, Oregano; 2, Catnip; 3, Sage; 4, Dark opal; 5, Thyme; 6, Basil; 7, Hyssop; 8, Peppermint; 9, Lamb's ear; 10, Lemon balm; ▩, 0.5%; ⬚, 2%. Different letters represent significant differences among treatments by Tukey's test ($P < 0.05$).

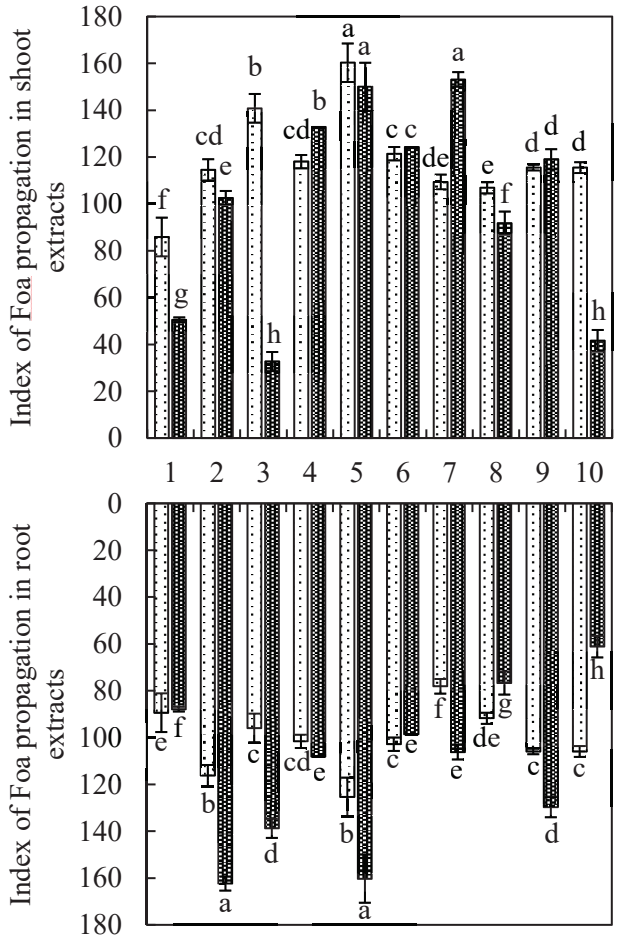


Fig. 10. Effect of herb extract on propagation of Foa (*Fusarium oxysporum* f. sp. *asparagi* ; MAFF305556). Here, 1, Oregano; 2, Catnip; 3, Sage; 4, Dark opal; 5, Thyme; 6, Basil; 7, Hyssop; 8, Peppermint; 9, Lamb's ear; 10, Lemon balm; □, 0.5%; ▩, 2%. Different letters represent significant differences among treatments by Tukey's test ($P < 0.05$).

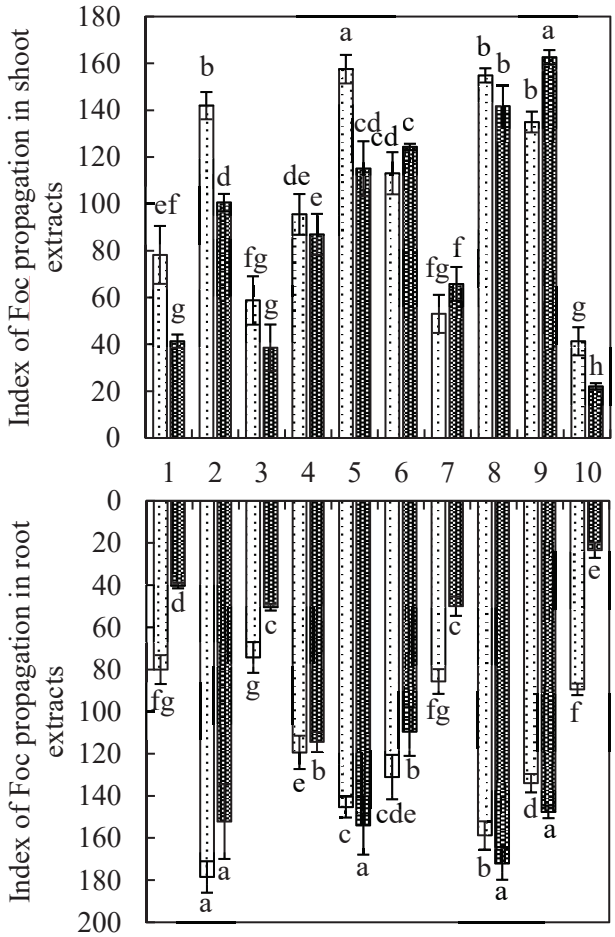


Fig. 11. Effect of herb extract on propagation of Foc (*Fusarium oxysporum* f. sp. *cyclaminis*, MAFF 712100). Here, 1, Oregano; 2, Catnip; 3, Sage; 4, Dark opal; 5, Thyme; 6, Basil; 7, Hyssop; 8, Peppermint; 9, Lamb's ear; 10, Lemon balm; □, 0.5%; ▩, 2%. Different letters represent significant differences among treatments by Tukey's test ($P < 0.05$).

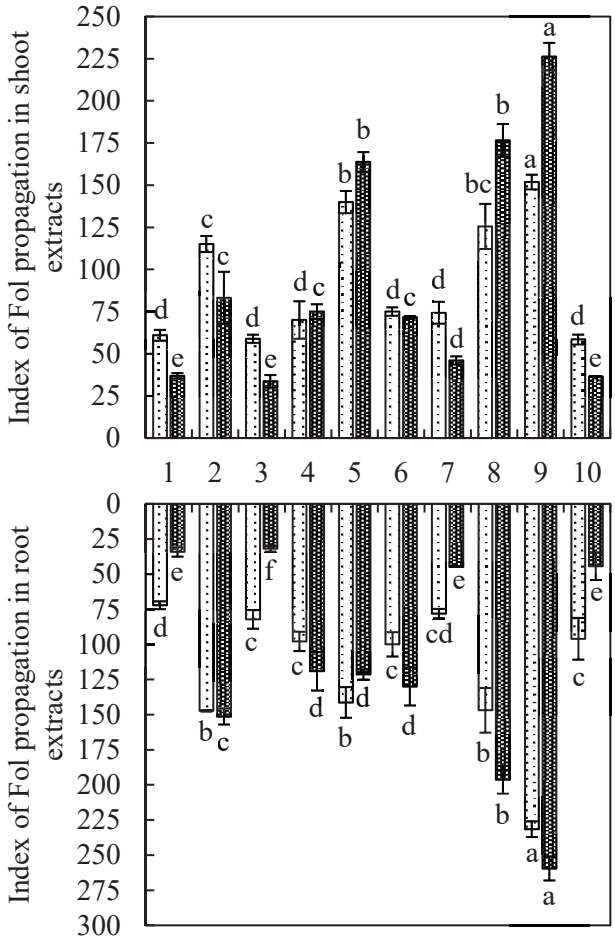


Fig. 12. Effect of herb extract on propagation of Fol (*Fusarium oxysporum* f. sp. *lycopersici*, MAFF238900). Here, 1, Oregano; 2, Catnip; 3, Sage; 4, Dark opal; 5, Thyme; 6, Basil; 7, Hyssop; 8, Peppermint; 9, Lamb's ear; 10, Lemon balm; □, 0.5%; ▩, 2%. Different letters represent significant differences among treatments by Tukey's test ($P < 0.05$).

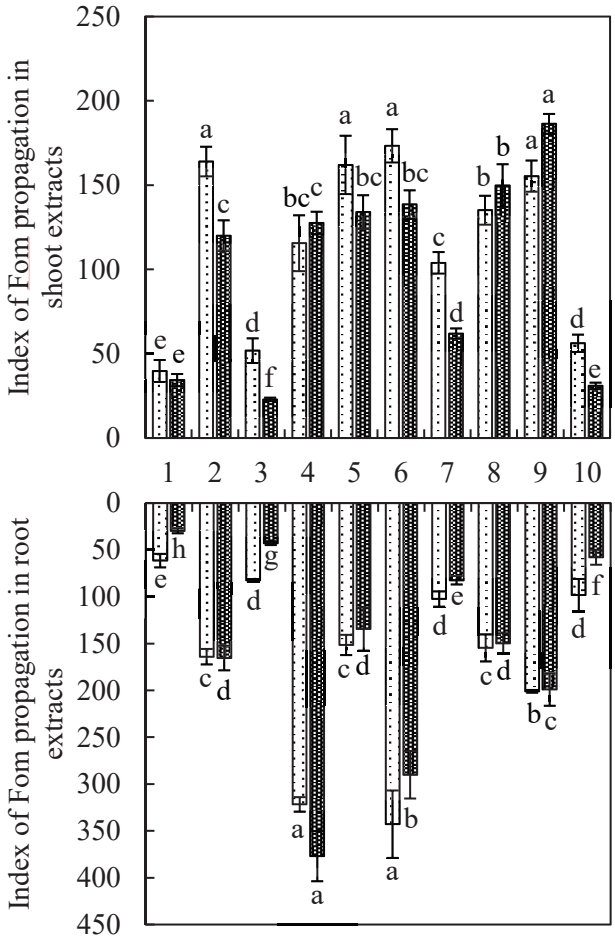


Fig. 13. Effect of herb extract on propagation of Fom (*Fusarium oxysporum* f. sp. *melonis*; MAFF242352). Here, 1, Oregano; 2, Catnip; 3, Sage; 4, Dark opal; 5, Thyme; 6, Basil; 7, Hyssop; 8, Peppermint; 9, Lamb's ear; 10, Lemon balm; □, 0.5%; ▩, 2%. Different letters represent significant differences among treatments by Tukey's test ($P < 0.05$).

Discussion

Plants has been the prime source of ingredients in traditional medicine since thousands of years ago. The curative and preventive activities of the plant parts and extracts have served as the basic foundation of the medicinal treatments of today. The recent progress in modern therapeutics has further influenced the use of plant based natural products against various diseases (Miraj et al., 2017). Among the different plant sources, herbs are considered to be an important source of therapeutic constituents. The mint family or *Lamiaceae* is one of the major medicinal plant branches that is commonly found in various agro-climactic conditions and are quite popular for their various uses (Carović-Stanko et al., 2016). Besides being used as culinary ingredients, in recent time, they are being used as a source of natural antioxidants to tackle many present-day obstacles faced by humans; both biotic and abiotic. ROS production in living organisms can cause disruption in cellular balance by overthrowing antioxidant defense leading to oxidative stress. On the other hand, synthetic antioxidants have the possibility to induce carcinogenesis as a negative impact (Suhaj, 2006). As such, importance of plant derived natural antioxidants are of utmost importance now. Most of the sources of antioxidants from the plants under *Lamiaceae* family are found from the sub-family Nepetoideae, which includes basil, lemon balm, marjoram, peppermint, oregano, rosemary, sage etc. (Albayrak et al., 2013; Carović-Stanko et al., 2016). The presence of phenolic acids and volatile terpenes were considered to be responsible for the antioxidative effects (Wink, 2003). Most studies had given emphasis on the EOs of these herbs and different organic distillation process (Wang et al., 1998; Dapkevicius et al., 1998; Triantaphyllou et al., 2001). However, these organic extracts contained the characteristic flavors of the herbs which are sometimes undesirable (Teissedre and Waterhouse, 2000). Thus, water extraction of these herbs could possibly eliminate this problem due to hydrophobic nature of the EOs. However, information regarding antioxidative properties of water extracts of *Lamiaceae* herbs are

scarcely found in literature. The results of the present study indicated that, the water extracts of these herbs also contain a considerable antioxidative properties as evidenced by the high DPPH radical scavenging activity. Among the 10 herbs that are evaluated, oregano sage, hyssop and lemon balm showed high radical scavenging activity compared to others. Higher DPPH activity by water extracts of these 4 herbs were also reported by Skotti et al. (2014). Capecka et al. (2005) also reported high DPPH scavenging potential of lemon balm and oregano water extracts. Presence of rosmarinic acid, an important phenolic acid that is commonly found in the herbs of *Lamiaceae* family is considered to be responsible for their radical scavenging activity (Lagouri and Alexandri, 2013). Particularly, oregano and lemon balm has been reported to contain a high amount of rosmarinic acid (Chen and Ho, 1997; Zgórka and Głowniak, 2001). Other common phenolic acids like caffeic acid, chlorogenic acid, ferulic acid etc. are also related to neutralization of this free radicle (Chen and Ho, 1997).

Polyphenol content of the herbs evaluated in this study showed a considerable variation among them. Oregano, sage, thyme, hyssop and lemon balm showed increased content compared to others. Contrary to our findings, aqueous extracts of lemon balm had been found to contain higher polyphenol content compared to oregano, sage, hyssop etc. (Katalinic et al., 2006; Fecka and Turek, 2007). On the other hand, some studies had reported oregano to contain high level of phenolic compounds (Kogiannou et al., 2013; Kaliora et al., 2014). This variation could be resulted due to different climatic conditions and growth situations (Skotti et al., 2014). The presence of high content of polyphenols could be related to the increased antioxidative ability of these herbs as numerous studies supports such correlation (Canadanovic´-Brunet et al., 2008; Li et al., 2008). Regarding ascorbic acid content, the results of the study showed that oregano, catnip, sage, hyssop, peppermint and lemon balm had a higher content compared to the other herbs. The high ascorbic acid content in fresh lemon balm, peppermint and oregano water extract was also reported by Capecka et al. (2005). He reported that, the decoctions

produced from fresh herbs were comparatively higher in ascorbic acid content compared to dried herbs. A close correspondence between ascorbic acid content and DPPH radical scavenging activity was also determined in previous studies (Hinneburge et al., 2006). As such, a high content of ascorbic acid could be an indicator of potentially strong antioxidative ability of extracts. From the results of the antioxidative ability, oregano, sage, hyssop and lemon balm extracts could be selected as the herbs with potentially high antioxidative effect among the 10 herbs considered.

Plant pathogenic fungi are one of the most infectious agents in plants that hampers the proper development of crops in different stages ultimately leading to plant death. Generally, the control measure against these pathogenic fungi is the use of synthetic fungicides. However, use of synthetic fungicides has the problem of causing harm to human health and environment due to their persistent presence and through entering food chain. As such, a search for suitable eco-friendly substitute of synthetic fungicides is the present concern of the researchers. Medicinal plants under the family *Lamiaceae* have long been used therapeutically against various diseases of human. More recently, interest has developed in use of these herbs extracts to control phytopathogens. However, information regarding the antifungal activity of water extracts of these herbs is quite scarce. From the results of the present study, it was quite evident that, the water extracts of these herbs also have the ability to suppress fungal pathogens *in vitro*. Especially, oregano, sage and lemon balm extracts showed considerable suppressive effect against all the Fusarium species considered in the study. Hyssop extract did not show suppressive effect on Foa although the rest were suppressed by it. The antimicrobial properties of plant extracts from various species were reported to prevent the growth of phytopathogens both *in vitro* and *in vivo* (Bautista-Baños et al., 2003). Beside shoot extracts, root extracts activity against soil born fungal species had also been reported (Ushiki et al., 1998). Most of these reports emphasizes the antimicrobial and preservative properties of the EOs of herbs and

are well documented (Martino et al., 2009, Soylu et al., 2010). However, the findings of our study showed that the water extracts of these herbs also possess direct antifungal activity. This antifungal activity of the herb water extracts was reported to be resulted by the presence of several phenolic compounds that acts as phytoalexins in the extracts. As such, a possible presence of such active compounds in the water extracts of these herbs might have resulted in the suppression of propagation. However, identification of such compound's presence will be needed to confirm such hypothesis.

Chapter 1- Conclusion

From the findings of the study, it can be concluded that, the water extracts from shoots and roots of herbs under *Lamiaceae* family expressed a varying degree of antioxidative ability with different levels of ascorbic acid and polyphenol content. Furthermore, the antifungal evaluation of the herb water extracts against several Fusarium species showed a considerable suppression ability especially from oregano, sage, hyssop and lemon balm. Considering the results, it can be proposed that, these 4 herbs have the potential to suppress several fungal pathogens *in vitro* which also have a considerable antioxidative effect. However, many times the suppression observed in *in vitro* analysis is often not observed *in* vivo. Further research regarding their applicability in practical condition as well as identification of the antifungal components of the extracts is necessary to establish such hypothesis. We tried to address these points in the following chapter.

CHAPTER 2-1

Effect of lemon balm water extract on Fusarium wilt control in strawberry and antifungal properties of secondary metabolites

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 (expressed highest suppression activity against Fof *in vitro*) on Fusarium wilt control in strawberry *in vivo*, 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 Co. Ltd., 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 (50mL/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 10^6 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 $\mu mol \cdot m^{-2} \cdot s$) 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:

$$\text{Disease index} = \frac{\sum(\text{number of plants} \times \text{number of degree in symptoms})}{\text{Total number of plants} \times \text{maximum degree in symptoms}} \times 100$$

Ten plants from each treatment were seperated 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 (Table 1), 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 (Nihon Pall Ltd. Tokyo, Japan) to remove proteins in the extract.

The samples were analyzed 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 thermostation 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) analyzed 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) (Table 2) 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 (10^6 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$\times 10^4$

Statistical analysis: Mean values were analyzed 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).

Table 1. Composition of Komada's medium.

Chemicals/compounds	Quantities (g/l)	Remarks
K_2HPO_4	1.0	
KCl	0.5	
$MgSO_4 \cdot 7H_2O$	0.5	
Fe-Na-EDTA	0.01	
L-asparagine	2.0	Pentachloronitrobenzen, $Na_2B_4O_7 \cdot 10H_2O$, cholic acid sodium salt and streptomycin sulphate were added after the medium was autoclaved in 1.0 lit, of distilled water and cooled. Finally, pH was adjusted to 3.8 ± 0.2 with 10 % H_3PO_4.
D-glucose	20.0	
Agar	15.0	
Pentachloronitrobenzene	1.0	
$Na_2B_4O_7 \cdot 10H_2O$	1.0	
Cholic acid sodium salt	0.5	
Streptomycin sulphate	0.3	

Table 2. Composition of Czapek-Dox liquid media.

Chemicals/compounds	Quantities (g/l)
$NaNO_3$	3.0
KH_2PO_4	1.0
$MgSO_4 \cdot 7H_2O$	0.5
KCl	0.5
$FeSO_4 \cdot 7H_2O$	0.01
Sucrose	30.0
pH	5.8

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. 14). In the control plants, the incidence of Fusarium wilt in shoots reached 100%, with 50% of plants scored as severity level 5 (Fig. 15A). 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. 15A). 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 (Fig. 16). 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. 15B).

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. 17). CFUs in the soil of plants treated with lemon balm extract were below 5×10^4, while in the control they were as high as 26×10^4.

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. 18A, 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. 19A 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. 19B, D) at a similar retention time (Fig. 19C 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. 20). The Fof populations in the media containing rosmarinic acid, luteolin, and caffeic acid were considerably lower (66×10^4, 51.3×10^4, and 65.7×10^4 CFU/mL, respectively) than those in the control (131×10^4 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.

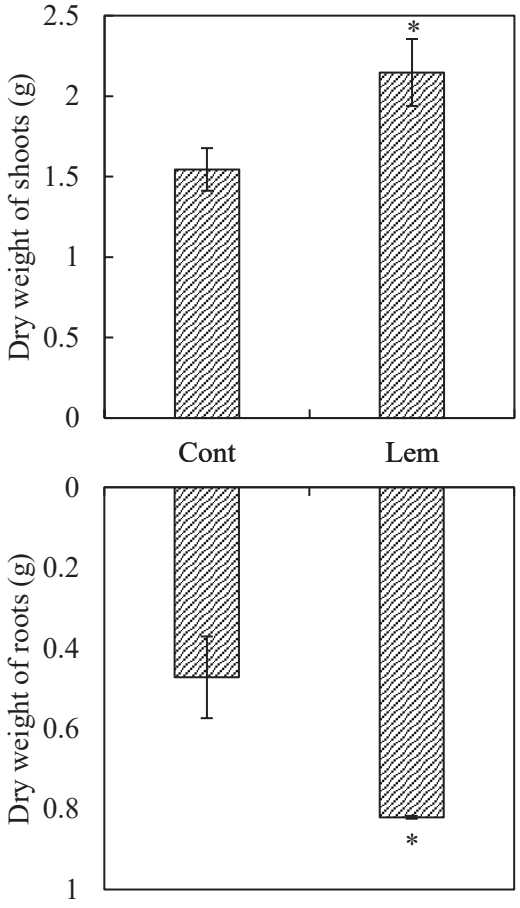


Fig. 14. Dry weight of shoots and roots of strawberry under herb extract treatment after Fof inoculation. Cont, control; Lem, treated with lemon balm shoot extract. *, significant difference according to *t*-test ($P < 0.05$).

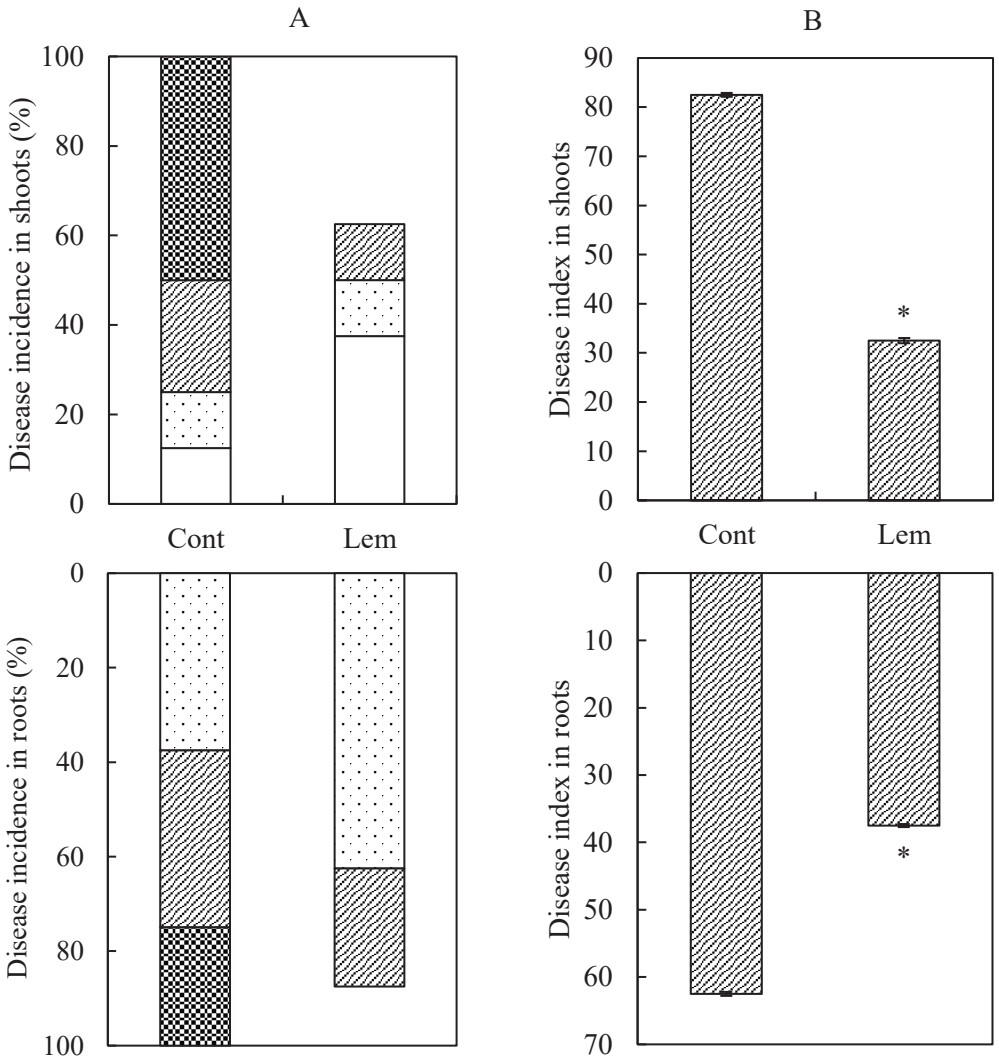


Fig. 15. Incidence (A) and index (B) of Fusarium wilt in shoots and roots of strawberry plants. Cont, control; Lem, treated with shoot extract of lemon. *, significant difference according to t-test ($P < 0.05$). For shoots: □, 0–20; ⁘, 20–40; ▨, 40–60; ▩, 60–80; ■, 80–100%. for roots: ⁘, a part diseased; ▨, half diseased; ■, all diseased.

Cont Lem

Cont Lem

Fig. 16. Effect of lemon balm extract on Fusarium wilt of strawberry. Here, Cont, control plants; Lem, plants treated with lemon balm shoot extract.

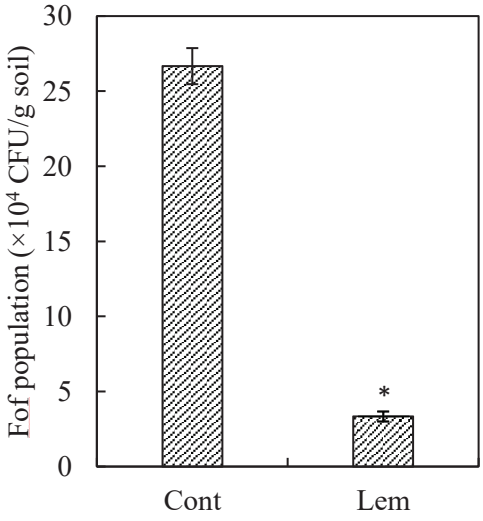
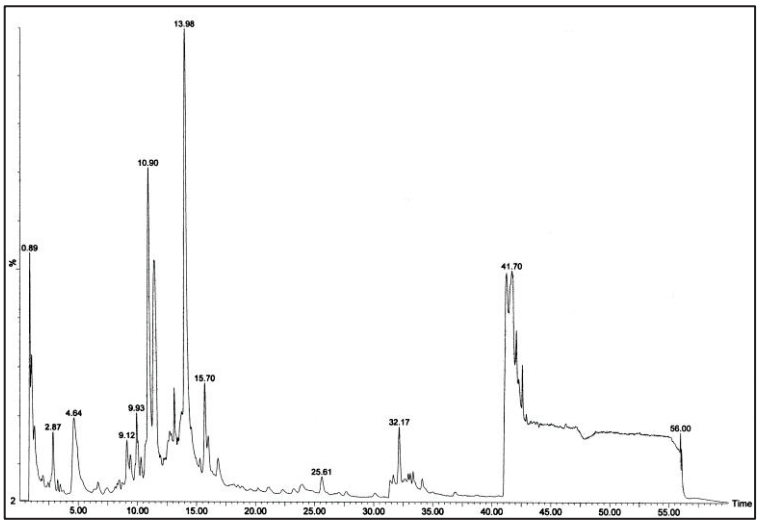


Fig. 17. Effects of lemon balm extract on Fof population in rhizospheric soil. Cont, control; Lem, treated with shoot extract of lemon balm. *, significant difference according to *t*-test ($P < 0.05$).

A



B

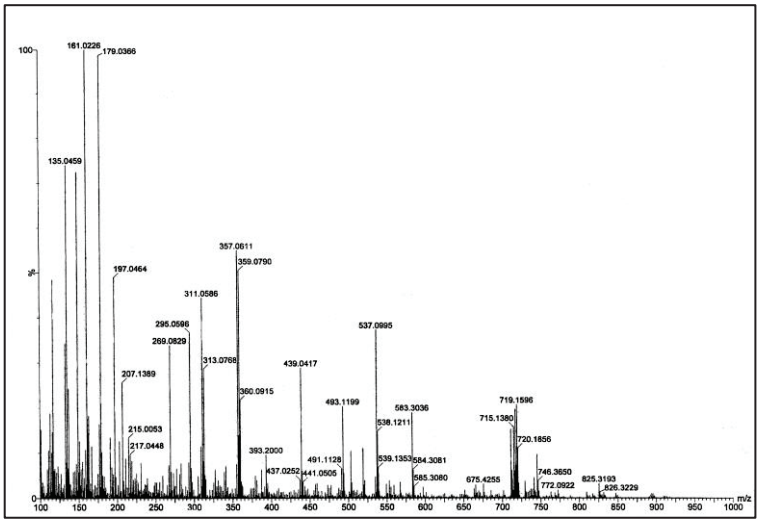


Fig. 18. UPLC-MS analysis of lemon balm water extract and identification of rosmarinic acid in lemon balm water extract by chromatogram and MS spectrum of the collision fragments using LC-MS/MS. A, chromatogram of lemon balm extract; B, spectrum of lemon balm extract.

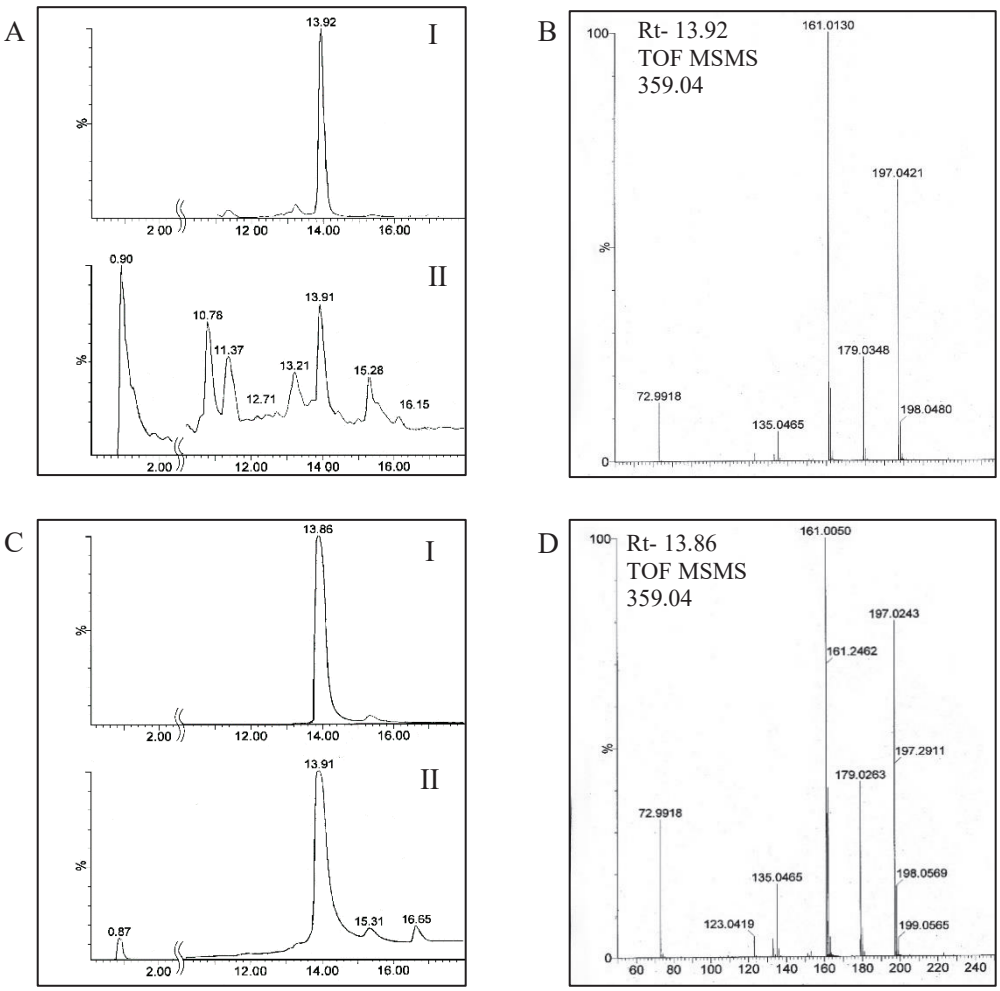


Fig. 19. Confirmatory UPLC-MS analysis of lemon balm water extract for rosmarinic acid. A (I) selected retention time of the extract; (II) LC data of the herb extract; B, MS/MS collision spectrum at 13.92 min; C (I) selected retention time of rosmarinic acid solution; (II) LC data of standard rosmarinic acid; D, MS/MS collision spectrum at 13.86 min.

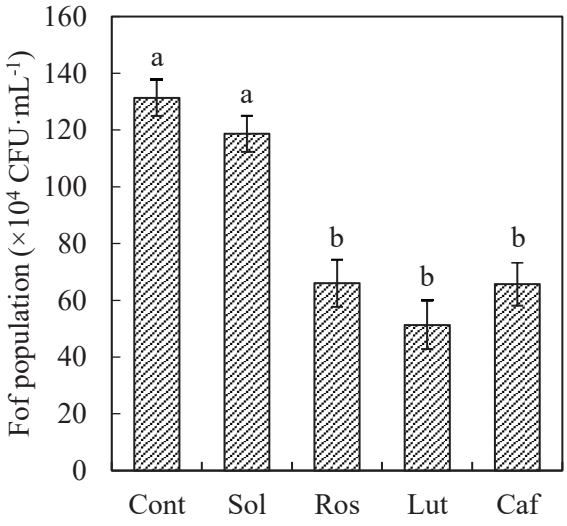


Fig. 20. Effects of identified chemical compounds on Fof propagation *in vitro*. Cont, control; Sol, solvent (distilled water:ethanol = 24:1, v/v); Ros, rosmarinic acid; Lut, luteolin; Caf, caffeic acid. Columns denoted by different letters indicate significant differences based on Tukey's test ($P < 0.05$).

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 suppress 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 soluble in polar solvents such as water (Tommasini et al., 2004), which could be the reason for the fluctuating concentrations of luteolin in the supplemental experiments. Here, the identified compounds exhibited strong suppressive effects against Fof propagation *in vitro*. Therefore, it can be stated that the synergistic action of rosmarinic acid, luteolin, and caffeic acid present in lemon balm extract conferred antifungal properties against Fof. In addition, the *in vitro* test revealed that the compounds individually exhibited similar suppressive effects on Fof. Nevertheless, among the three metabolites, rosmarinic acid could be the major contributor to the antifungal properties of the extract owing to its stability and 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 are 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 characteristic 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. For this evaluation, lemon balm was selected as it showed the highest suppression rate against Fof in *in vitro* evaluation presented in the chapter 1. The suppression observed *in vitro* was also found in the bioassay that confirmed its potential use in practical production system. The next section of chapter 2 addressed the applicability of herb extract in suppression of top-part disease of strawberry such as anthracnose to see whether the herb extracts could provide us a way to get dual suppression against both root and shoot diseases of strawberry.

CHAPTER 2-2

Suppression of anthracnose in strawberry using water extracts of *Lamiaceae* herbs and identification of antifungal metabolites

Introduction

The organisms belonging to genus *Colletotrichum* are important plant pathogens that cause anthracnose in a wide range of plants worldwide (Cannon et al., 2012). In strawberry cultivation, *C. gloeosporioides* is a serious disease-causing organism causing huge production loss in major strawberry producing regions (Mori and Kitamura, 2003). It can infect several parts of strawberry plants, and its symptoms include crown and stolon necrosis (Howard et al, 1992), black leaf spot (Howard and Albregts, 1983), and fruit lesions (Howard and Albregts, 1984). It can also affect the production cycle causing up to 60-70% of yield loss (Legard et al., 2003; Smith, 2008). However, it is difficult to control the disease as mother plants with latent infection are often used for runner production. Moreover, the difficulty in developing cultivars due to polyploidy, incomplete resistance of the developed cultivars and inadequate control through cultural control methods makes this disease a serious problem in strawberry cultivation. Generally, the use of synthetic fungicides is the primary control measure used against this disease at the producer level. However, these chemicals pose a major threat to the environment as well as to humans because of their low selectivity and lack of biodegradability (Gao et al., 2017). Furthermore, the development of resistance by the microorganisms to these chemical compounds results in higher dose dependence, which increases the production cost as well as food safety problems (Jílková et al., 2015). Hence, a search for alternative and environment-friendly approach for disease control has become the present challenge in crop production.

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 herbs are well documented, primarily for agri-foods (Teixeira et al., 2013; Gomes et al., 2014). In addition, the *in vitro* antioxidant and

antifungal effects of the EOs on plant pathogens have also been reported in a few studies (Isman 2000; Quintanilla et al., 2002). However, the antifungal effects of *Lamiaceae* herbs on plant disease control remain unclear.

Lemon balm (*Melissa officinalis* L.) of the *Lamiaceae* family is an important medicinal herb that has been widely used in traditional medicines (Meftahizade et al., 2010); its essential oil is reported to possess antimicrobial activity (Romeo et al., 2008), whereas its aqueous extract is reported to exhibit antiviral (Adorjan and Buchbauer, 2010), antioxidant (Spiridon et al., 2011), anti-inflammatory, antinociceptive (Birdane et al., 2007), and antidiabetic effects (Chung et al., 2010). On the contrary, the use of oregano (*Origanum vulgare* L.) has only increased in recent years because of the identification of several therapeutic properties of its extract such as, antioxidant, antimicrobial, anti-inflammatory (Oniga et al., 2018), antiviral (Zhang et al., 2014), antispasmodic (Goncearіuc et al., 2015), antiproliferative (Elshafie et al., 2017), and neuroprotective (Gîrd et al., 2016) effects. Quintanilla et al. (2002) reported that the EOs of herbs such as thyme, oregano, lemon balm, and peppermint inhibited the growth of *Phytophthora infestans* in the *in vitro* plate assay. In addition, the EOs of lavender and rosemary were found to suppress 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 inhibiting the fungi. However, the use of EOs in field conditions is impractical because they would diffuse away from the applied surface, resulting in a decrease in the effective concentration, which would enable the disease-causing organism to resume growth (Letessier et al., 2001). In addition, EOs have been reported to possess phytotoxic effects in crops following foliar application at high concentrations (Letessier et al., 2001). A viable alternative to this could be the use of water extracts containing non-volatile secondary metabolites, in pursuit of environmentally-friendly disease control approaches. Water extract preparation is a relatively easy and inexpensive process compared with that of

EOs. In addition, as the extracts are non-volatile, they remain effective for longer periods than the EOs. The effectiveness of lemon balm water extract in controlling Fusarium wilt in strawberry has already been confirmed in our previous study (Ahmad and Matsubara, 2019, in press) with the water extract showing considerable suppression of the fungal propagation leading to lower disease incidences and indices; the secondary metabolites identified in the extract also suppressed the pathogen *in vitro*, thereby proving their antifungal potential. Thereupon, we decided to test see whether the extracts of *Lamiaceae* herbs can also suppress diseases of strawberry affecting its upper parts such as anthracnose. Therefore, in this study, we evaluated the effect of water extracts of lemon balm and oregano on controlling anthracnose in three strawberry cultivars. We also identified the major secondary metabolites present in these water extracts and evaluated their antifungal potential.

Materials and Methods

Growing *Lamiaceae* herbs and preparing their extracts: Seeds of *M. officinalis* and *O. vulgare* were sown in plastic containers (31.9 cm × 26.4 cm × 15.3 cm) containing commercial soil (Supermix A, Sakata Co. Ltd., Japan) and grown in the greenhouse. Eight weeks after sowing, the plants were uprooted and the shoots were cryopreserved using liquid nitrogen. The 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 then filtered and the filtrate was used as the herb water extract.

Bioassay of herb extracts for Anthracnose control in strawberry: Strawberry (*Fragaria* × *ananassa* Duch.) runner plants of three cultivars (‘Sachinoka,’ ‘Akihime,’ and ‘Tochiotome,’ susceptible to anthracnose) were grown in pots (10.5 cm in diameter, 0.5 L) containing autoclaved commercial soil (SM-2, Premier Tech., Canada) and fertilized using slow-releasing granular fertilizer (Long Total 70 day type; N:P:K = 13:9:11, JCAM Agri. Co. Ltd., Japan).

After four weeks, the water extracts (20%, w/v) of lemon balm and oregano leaves were sprayed (10 ml/plant) on the strawberry plants two times before pathogen inoculation. For control plants, distilled water was used. *C. gloeosporioides* was cultivated on potato dextrose agar medium and incubated in dark conditions at 25 °C for two weeks to facilitate sporulation; they were further subcultured for 7–10 days to facilitate more sporulation. The spores were harvested in distilled water and the concentration was adjusted to 10^5 conidia/ml. Each strawberry plant of the three cultivars was sprayed with 10 ml of the conidial suspension immediately after the second spraying of the herb extracts, and they were covered with plastic films for the first week to maintain humid condition around them to facilitate inoculation (28 ± 3 °C). Ten plants per treatment in triplicates were grown in a growth chamber at 28 ± 3 °C with a 12 h photoperiod (750-1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and 70-80% relative humidity. Two weeks after inoculation, the symptoms of anthracnose were assessed as described by Li et al. (2010), i.e., percentage of diseased leaves and petioles using 5 levels: 1 Level 1, 0<□<20%; Level 2, 20<□<40%; Level 3, 40<▨<60%; Level 4, 60<▩<80%; Level 5, 80<■<100%. The disease index was calculated using the following formula:

$$\text{Disease index} = \frac{\sum (\text{number of plants} \times \text{number of degree in symptoms})}{\text{Total number of plants} \times \text{maximum degree in symptoms}} \times 100$$

Ten plants were randomly chosen from each treatment and separated to shoots (compatible leaves and petioles), crown and roots. The roots were cleaned very carefully under slow flowing tap water in a tray to remove soil and debris and prevent loss of fine roots. After that, they were dried using a constant temperature drier (ETTAS 600B) at 80 °C for 2 days. The dry weights of shoots and roots were then measured.

Analysis of herb water extracts using UPLC-MS/MS: From the cryopreserved samples of five plants, 0.6 g of lemon balm and oregano leaves were pulverized separately in a mortar

with liquid nitrogen to a fine powder and mixed with 3 ml of ultrapure water to prepare a sample extract solution (20%, w/v) for each. The sample solutions were then centrifuged at 13,000 rpm for 15 min at 4 °C using Nanosep 10K (Pall Corporation, Tokyo, Japan) to remove proteins in the extracts; the supernatants were filtered through a sterilizing filter (0.45 µm, ADVANTECH Co. Ltd., Japan).

The samples were analyzed 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 of 0.1% formic acid in water (A) and acetonitrile (B) at a flow rate of 0.4 ml/min. The sample injection volume was 10 µl. The gradient profile was as follows: 0–6 min, 95% A; 6–12 min, 75% A; 12–30 min, 65% A; 30–32.5 min, 5% A; and 32.5–35 min, 95% A. The mass range of electrospray ionization was analyzed in negative mode at 50-1000 m/z using a mass spectrometer (Xevo Q Tof MS, Waters Corporation, Milford, USA), and the MS/MS collision was performed at 30 V. A mass chromatogram of the m/z value of each component in the extract was prepared from the measurements obtained using the retention time.

Evaluation of identified compound for antifungal effect against *C. gloeosporioides*: The four major compounds identified through UPLC-MS/MS analysis were evaluated for their efficacy against *C. gloeosporioides* using the poisoned food technique (Gupta and Tripathi, 2011). Two milligrams of rosmarinic acid, luteolin (both identified in the water extract of lemon balm), protocatechuic acid, and apigenin (both identified in the water extract of oregano) were separately dissolved in 40 µl of ethanol, and 960 µl of distilled water was added to each of the four solutions. Thereafter, 1 ml of each of these solutions was mixed with 19 ml of Czapek-Dox agar media (Table 3) separately and poured into 9-cm sterile Petri dishes; distilled water was used as control. After solidification of the media, a mycelial disk (5 mm) of 1-week old *C. gloeosporioides* was cut using cork borer and placed on the center of the media of each

petri dish. The experiment was performed in triplicates for each compound. The inoculated plates were then incubated at 28 °C and diameters of the colonies were measured every day for 7 days. The percent inhibition for each compound was determined using the following formula:

$$\text{Percent inhibition} = \frac{X - Y}{X} \times 100$$

Where X: diameter of fungal colony growth on control plate and Y: diameter of fungal colony growth on plates containing identified compounds.

Statistical analyses: The mean values for the dry weights of shoots and roots and antifungal effects of the identified compounds were analyzed by Tukey's multiple range test at $P < 0.05$. As the data for disease index was non-normally distributed, it was analyzed using Steel–Dwass multiple range test ($P < 0.05$). All the analyses were conducted using XLSTAT 2012 pro statistical analysis software (Addinsoft, New York).

Table 3. Composition of Czapek-Dox agar media.

Chemicals/compounds	Quantities (g/l)
$NaNO_3$	3.0
KH_2PO_4	1.0
$MgSO_4 \cdot 7H_2O$	0.5
KCl	0.5
$FeSO_4 \cdot 7H_2O$	0.01
Sucrose	30.0
Agar	8
pH	5.8

Results

Two weeks after *C. gloeosporioides* inoculation, the strawberry plants treated with herb extracts showed a significant increase in their shoot dry weight compared to that of control except for 'Akihime' under oregano treatment (Fig. 21). No significant difference was observed between the two herb treatments in case of shoot dry weights. The dry weights of roots increased in both the herb treatments for 'Sachinoka' and 'Tochiotome' compared to that of the control; however, in 'Akihime,' the control plants had a higher dry weight of roots compared to that of both the extract-treated plants. Similar to shoot dry weight, no significant difference was observed between the two herb treatments in case of root dry weights.

The incidence of anthracnose in control plants reached 100% in all the cultivars except for 'Akihime' and 25% of plants in all the cultivars expressed a severity level of 5 (Fig. 22A). On the contrary, plants treated with oregano exhibited lower disease incidence compared to that of control except for 'Akihime' with a lower severity level in all cultivars. The lowest disease severity (level 1) in all the cultivars was observed in plants treated with lemon balm; the disease incidence was also considerably low in lemon balm treated plants compared to control plants (Fig. 23). Both the herb extracts exhibited a significant decrease in their disease indices compared to that of the control with the lowest observed in lemon balm treated plants in all the cultivars (Fig. 22B).

The analysis of water extracts of lemon balm and oregano was conducted using UPLC-MS/MS and represented in the form of chromatograms and spectrum graphs (Fig. 24, 25). From the chromatograms, the most promising region of compounds with high peaks was observed between the retention time of 5 and 15 min. In the case of lemon balm extract, compounds with m/z values of approximately 359 and 295 were observed pertaining to the two major peaks found at 12.29 min and 10.47 min, respectively (Fig. 24A, B). After cross-referencing this data

with the mass bank (https://massbank.eu/MassBank/), the compounds were identified to be rosmarinic acid and luteolin. Another peak at a retention time of 9.81 min was observed, which appeared to be a fragment of the compound rosmarinic acid, however, its content was lower than that of the compound at 12.29 min (Fig. 24A). Oregano extract exhibited two highest peaks at 10.05 min and 11.30 min with the corresponding m/z values of approximately 269 and 153 (Fig. 25A, B). After comparing this data with the mass bank, the compounds were identified to be apigenin and protocatechuic acid. All these four compounds were found to be stable and present in high amounts than the other compounds present in the extracts, therefore, they were selected for *in vitro* antifungal evaluation against *C. gloeosporioides* in the present study.

The antifungal evaluation of the major components identified from lemon balm and oregano against *C. gloesporioides* showed promising results as observable from fig. 26. Among the four identified compounds from the two herb extracts, rosmarinic acid (61.7%) exhibited maximum growth suppression, which was statistically similar to the values exhibited by apigenin and protocatechuic acid (58.7 and 58.4%, respectively). Although luteolin showed lower suppression among the four compounds, it was still able to suppress mycelial growth close to 50%.

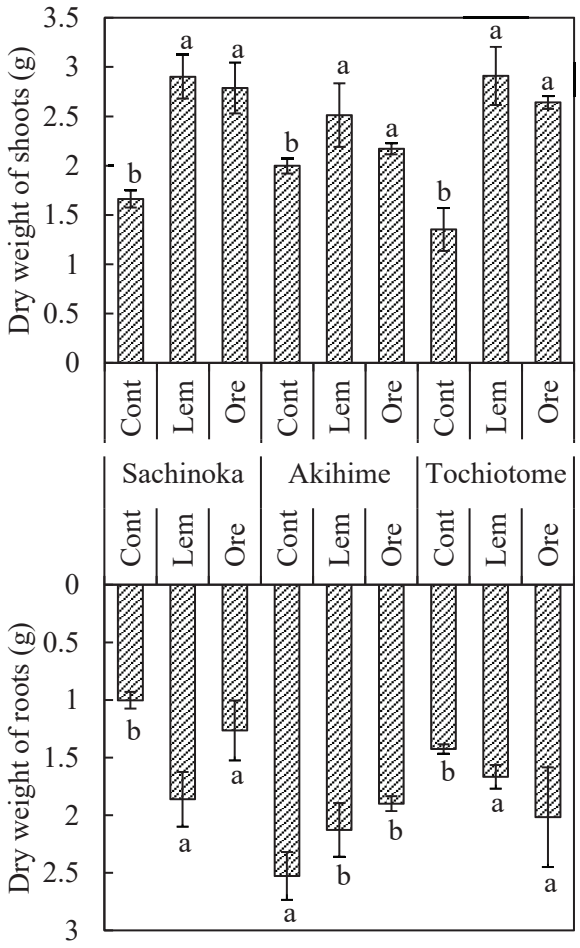


Fig. 21. Dry weight of shoots and roots of strawberry plants treated with lemon balm and oregano shoot extracts after CG1 inoculation. Cont, control; Lem, lemon balm; Ore, oregano. Columns denoted by different letters indicate significant differences according to Tukey's test ($P < 0.05$).

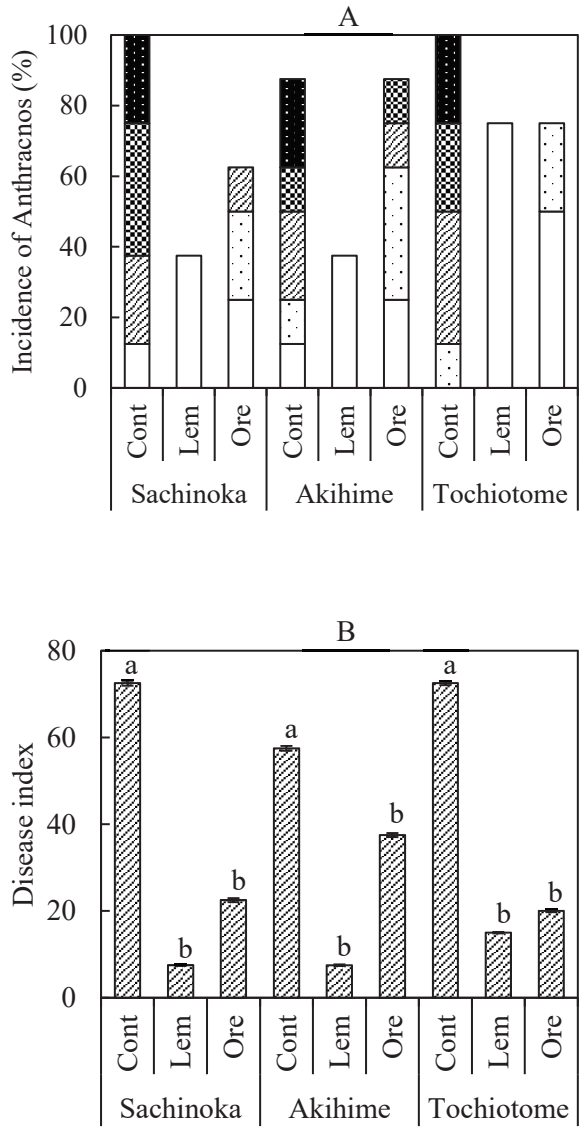


Fig. 22. Inhibitory effects of lemon balm extract on disease incidence (A) and indices (B) in strawberry plants after CG1 inoculation. Cont, control; Lem, lemon balm; Ore, oregano. Columns denoted by different letters indicate significant differences according to Tukey's test ($P < 0.05$). □, < 20%; ⊡, 20–40%; ▨, 40–60%; ▩, 60–80%; ■, 80–100%.

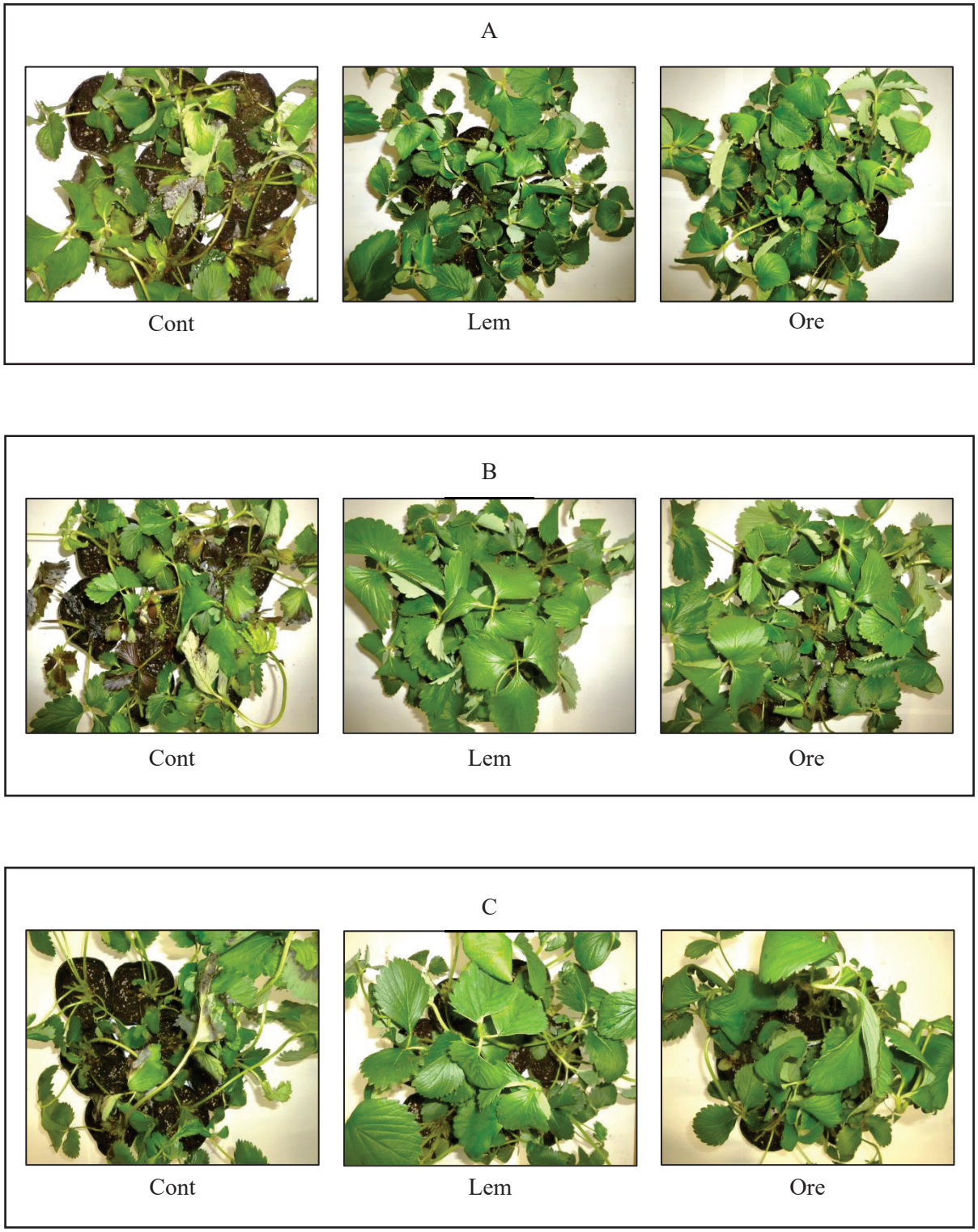
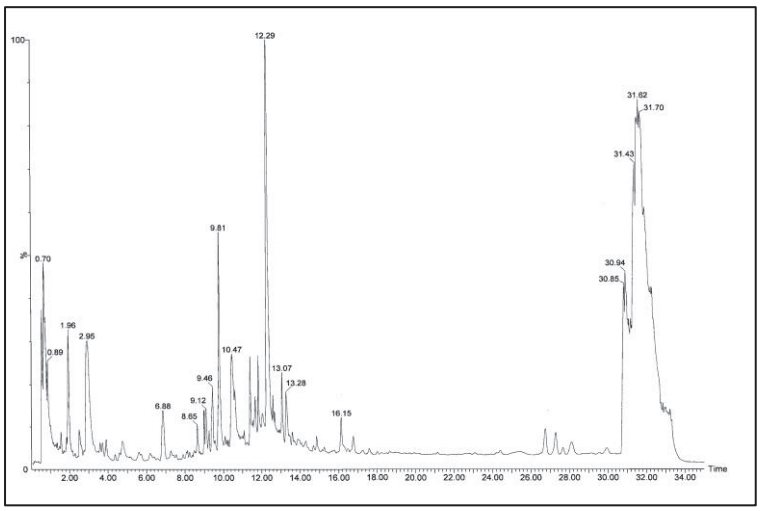


Fig. 23. Effect of herb extracts on anthracnose of strawberry. Here, A, Sachinoka; B, Tochiotome; C, Akihime; Cont, control plants; Lem, lemon balm; Ore, oregano.

A



B

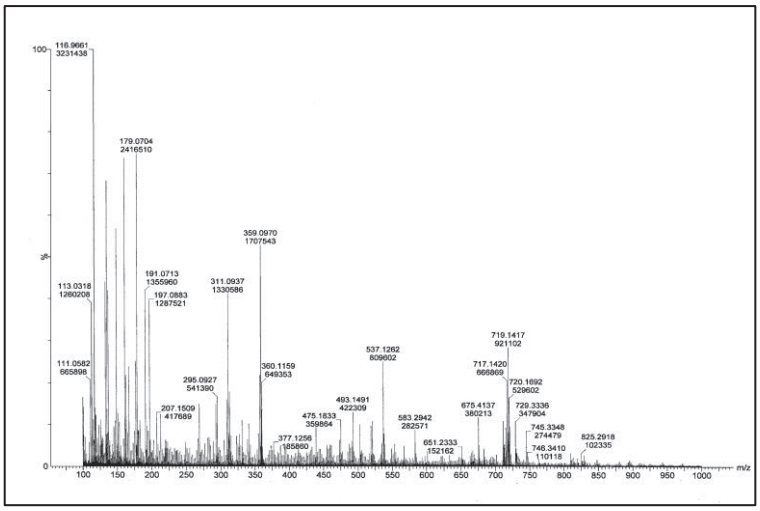
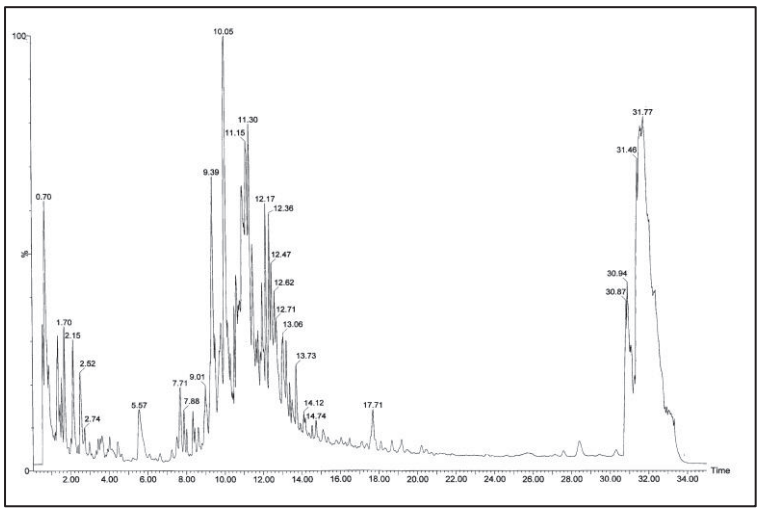


Fig. 24. Chromatogram (A) and mass spectrum (B) of lemon balm water extract derived by UPLC-MS.

A



B

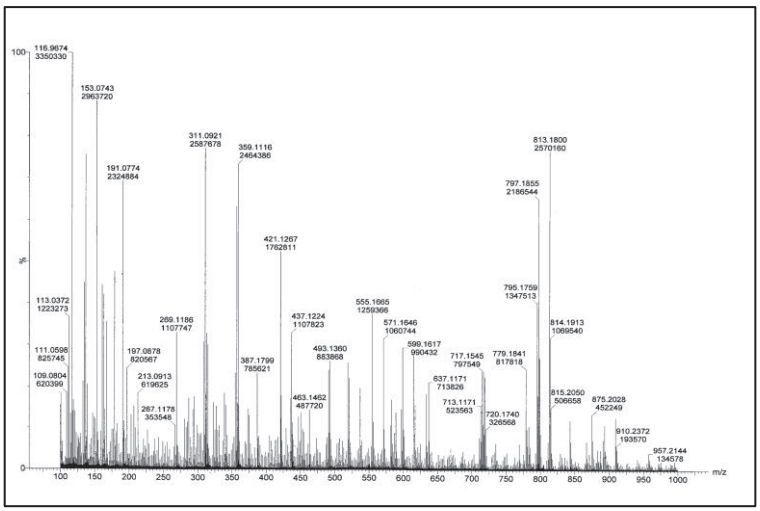


Fig. 25. Chromatogram (A) and mass spectrum (B) of oregano water extract derived by UPLC-MS.

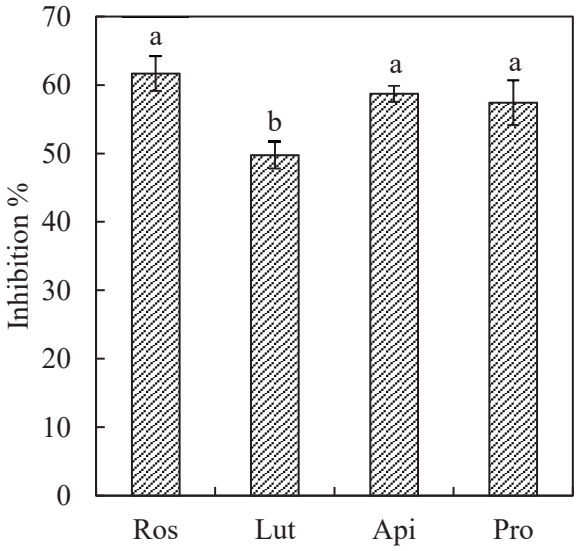


Fig. 26. Effects of identified chemical compounds on CG1 growth inhibition *in vitro*. Here, Ros, rosmarinic acid; Lut, luteolin; Api, apigenin; Pro, protocatechuic acid. Columns denoted by different letters indicate significant difference based on Tukey's test ($P < 0.05$).

Discussion

Lamiaceae herbs contain a large number of phenolic compounds with antibacterial, antifungal, and antiviral properties (Bais et al., 2002). However, most of the reports on the activities of herb extracts were from *in vitro* studies. Although, *in vitro* studies are critical in the identification of plant extracts with potential agricultural applications, *in vivo* evidence is required for their adoption for commercial use (Gorris and Smid, 1995). In the present study, the incidence of anthracnose was considerably lower in all the strawberry cultivars under the herb treatments compared to that of the control. Even though the cultivars tested were quite susceptible to anthracnose disease, the application of herb extracts prevented the disease from reaching higher severity levels as compared to that of the control. Relevantly, the disease indices were also lower in herb-treated strawberry plants. In addition, disease suppression also led to the healthy development of the plants as observed by higher dry weights of their shoots and roots. Lemon balm and oregano extracts have already been reported to possess antimicrobial and preservative effects (Abdellatif et al., 2014, Oliva et al., 2015); however, these activities were attributed to the volatile EOs present in the herbs (Quintanilla et al., 2002; Vardar-Unlu et al., 2003; Soylu et al., 2010). On the contrary, in the present study, we showed that water extracts of the herbs also have the potential of suppressing the disease-causing organisms.

The analysis of lemon balm and oregano water extracts using UPLC-MS/MS was represented as chromatograms and the two highest peaks and their corresponding m/z values were cross-referenced with mass bank; the two compounds pertaining to the two highest peaks were identified to be rosmarinic acid and luteolin in the lemon balm extract and apigenin and protocatechuic acid in the oregano extract. The presence of rosmarinic acid in lemon balm has been reported previously in various studies (Tóth et al., 2003, Miron et al., 2013), and its content was found to be higher in lemon balm than that in most other *Lamiaceae* herbs (Zgórka

and Głowniak, 2001). The presence of luteolin in lemon balm has also been reported in several studies (Patora and Klimek, 2002, Ordaz et al., 2018,) and it is one of the common flavonoids found in both methanolic as well as aqueous extracts. The presence of rosmarinic acid and luteolin in water extract of lemon balm was also confirmed in our previous study (Ahmad and Matsubara, 2020a). In addition, the presence of apigenin and protocatechuic acid has also been reported in several species of oregano and is common in both aqueous and non-aqueous extracts (Gutiérrez-Grijalva et al., 2017). The high content of these four compounds in lemon balm and oregano, respectively, makes them the possible principal components of the extracts responsible for suppressing the disease in this study; therefore, to confirm their antifungal activities, we performed further *in vitro* antifungal assays.

The four identified compounds showed considerable suppression of *C. gloeosporioides* during *in vitro* antifungal assay. It has been hypothesized that phenolic acids such as rosmarinic acid act as phytoanticipins in plants (Dixon, 2001). Bais et al. (2002) reported that the antifungal activity of rosmarinic acid is exerted through the breakage of the interseptum of the fungal mycelia and damage to the fungal cell surface by pilferage. Such specific activity of rosmarinic acid against microorganisms makes it a potent and novel antimicrobial agent. The flavonoids such as luteolin and apigenin have been reported to possess membrane disruption ability (Górniak et al., 2019). Ollila et al. (2002) reported that flavones such as apigenin cause destabilization of the membrane structure by disordering and disorienting the membrane lipids inducing leakage. Furthermore, flavonoids have also been reported to cause bacterial aggregation by partially lysing them, leading to membrane fusion and consequently reducing the active nutrient uptake via a smaller membrane (Górniak et al., 2019). Apigenin was also found to have antibiofilm formation activity against bacteria (Awolola et al., 2014). Xie et al. (2014) also linked the antimicrobial effects of flavonoids to their capacity to form complexes with extracellular and soluble proteins and with the cell wall. Luteolin was reported to possess

antifungal activity against *Aspergillus niger*, *Trichophyton mentagrophytes*, and *Candida albicans* (Abad et al., 2007). Protocatechuic acid was also reported to have considerable antimicrobial activity against several gram-positive and -negative bacteria (Alves et al., 2013). Therefore, the presence of rosmarinic acid and luteolin in lemon balm extract as well as apigenin and protocatechuic acid in oregano extract could be responsible for the antifungal effect of the extracts. Furthermore, the compounds could have worked synergistically in suppressing the pathogen when applied as water extracts of the herbs, which suggest the possible potential of using water extracts as antimicrobial agents. Most importantly, combining the findings of our previous study (Ahmad and Matsubara, 2020a), which evaluated the effect of major compounds in lemon balm extract on *Fusarium oxysporum* f. ap. *fragariae*, and the present study, we can conclude that the major compounds in lemon balm extract especially rosmarinic acid and luteolin suppress the growth of both *F. oxysporum* and *C. gloeosporiodes*. Therefore, it can be hypothesized that lemon balm extract might exert a dual suppression effect against both these diseases. In the wake of severe detrimental effect of synthetic agrochemicals, it could potentially provide an economically viable solution to commercial farmers against not one but two major diseases; however, further studies are required to clarify this hypothesis. The absence of caffeic acid in the lemon balm extract in the present study is a stark contrast to our previous study and could be the result of fluctuating levels observed in supplemental analysis of the extract in our previous study (Ahmad and Matsubara, 2020a). However, it did not prevent the extract from exhibiting disease suppression due to the stable presence of rosmarinic acid and high content of luteolin. Moreover, the identified compounds of oregano also showed suppression of anthracnose in the study which presents an alternative choice of extract for use against this disease and shows promise of possible versatile applicability of different herb extract from *Lamiaceae* family.

The general application method adopted by farmers for controlling diseases such as anthracnose is usually by spraying the agrochemicals. However, the innate volatile nature of the EOs prevents their application by spraying in field conditions as these spontaneously evaporate from the applied surface, rendering them useless (Letessier et al., 2001). Conversely, the water extracts due to being nonvolatile would remain effective for a longer time which point to its practical use.

In the present study, the direct effects of the antifungal properties of lemon balm and oregano water extract on *C. gloeosporioides* and subsequent disease suppression were observed. From the findings, it can be concluded that, *Lamiaceae* herbs extracts has the potential to suppress major diseases of strawberry like anthracnose and can be utilized as an eco-friendly control measure in field condition. Combining the results of the chapter 2-1 and 2-2, it could be confirmed that the use of lemon balm water extract in strawberry production system could provide a dual protection against both Fusarium wilt and anthracnose disease. This provides the producers an eco-friendly approach in addressing both these serious diseases of strawberry simultaneously. The next two segments of chapter 2 evaluated the use of herb extracts on suppressing diseases of other important horticultural crops like asparagus and cyclamen to see whether similar phenomenon could be observed in those cases.

CHAPTER 2-3

Antifungal effect of *Lamiaceae* herb water extracts against Fusarium root rot in asparagus

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 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). However, the antifungal effects and antioxidative properties of *Lamiaceae* herbs on plant disease control remain unclear.

Asparagus decline is a serious and increasing threat in asparagus producing regions all over the world (Reid et al. 2002; Hamel et al. 2005; Elmer 2015). The cause of asparagus decline can be attributed to both biotic (Wong and Jeffries, 2006; Knaflewski et al. 2008) and abiotic factors (Yong 1984; Miller et al. 1991; Lake et al. 1993). Among biotic factors, the most prevalent phenomenon is the Fusarium crown and root rot caused by *Fusarium oxysporum* f. sp. a*sparagi* (Foa), *Fusarium proliferatum* (Fp), *Fusarium redolens* (Fr) etc. (Reid et al. 2002; Wong and Jeffries 2006; Knaflewski et al. 2008). According to Nahiyan et al. (2011), Foa and Fp are the dominant Fusarium species causing asparagus decline in Japan. The disease is quite difficult to control due to the perennial nature of the crop, the pathogen being soil born and lack of resistant cultivar (Pontaroli et al. 2000; Elmer 2015).

Upon pathogen invasion, high concentration of reactive oxygen species (ROS) gets produced in plants as a defense mechanism, a phenomenon known as oxidative burst. The concentration is usually higher than normal which could prove toxic to the invading pathogen. However, this mechanism is a double-edged blade as the excessive production of ROS could possibly overwhelm the plant's own antioxidant defense, causing cell damage (Vanacker et al.

1998). Reports about application of synthetic antioxidants to tackle this ROS production and inducing disease resistance are present (Gala and Abdou 1996; El-Gamal et al. 2007). However, information regarding potential use of natural antioxidant resources like *Lamiaceae* herbs and their extracts in this aspect is scarce.

According to Quintanilla et al. (2002), essential oils (EOs) of some herbs such as thyme (*Thymus vulgaris* L.), oregano (*Origanum vulgare* L.), lemon balm (*Melissa officinalis* L.) and peppermint (*Mentha piperita* L.) inhibited growth of *Phytophthora infestans* in *in vitro* plate assay. In addition, the EOs of lavender (*Lavandula stoechas* L. var. *stoechas*) and rosemary (*Rosmarinus officinalis* L.) 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 the EOs in field conditions becomes impractical because they would diffuse away from the applied surface, resulting in a decrease in the effective concentration, which would enable the disease-causing organism to resume growth (Letesseir et al. 2001). In addition, many of such extracts, particularly the EOs, have been reported to possess phytotoxic effects in crops following foliar application at high concentrations (Letesseir et al. 2001). A viable alternative could be the use of water extracts containing non-volatile secondary metabolites, in pursuit of environmentally-friendly disease control approaches. Water extract preparation is a relatively easy and inexpensive process compared with that of the EOs. In addition, as the extracts are non-volatile, they would remain effective for longer periods than the EOs. From the *in vitro* assay of 10 herbs against Foa (presented in chapter 1), oregano, sage and lemon balm shoot extracts showed the highest suppression effect. However, besides *in vitro* analysis, 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 with those observed *in vivo* (Benner 1993). In light of this, the following experiment was conducted to

evaluate the antifungal activity of these herbs water extracts against Fusarium root rot in asparagus through bioassay. Beside these three herbs, hyssop was also selected for the bioassay as it expressed high activity in antioxidative evaluation of the 10 herbs to see whether any secondary way of disease suppression could be envisioned.

Materials and Methods

Growing of Lamiaceae herb: Seeds of oregano (*Origanum vulgare* L.), sage (*Salvia officinalis* L.), hyssop (*Hyssopus officinalis* L.), and lemon balm (*Melissa officinalis* L.) were sown in plastic containers (31.9 cm × 26.4 cm × 15.3 cm) containing autoclaved commercial soil (Supermix A, Sakata Co. Ltd. Japan) and grown in a greenhouse. Eight weeks after sowing, the plants were uprooted and the shoots were cryopreserved using liquid nitrogen.

Bioassay of herb extract for Fusarium rot control in asparagus: For bioassay, frozen samples of the four selected herbs (oregano, sage, hyssop and lemon balm) were ground in distilled water separately using a mixer maintaining the concentration of the herbal extract to 200 mg/ml (w/v). The extracts were filtered and the filtrate was used as herb extract solution.

Seeds of asparagus (*Asparagus officinalis* L., cv. Welcome) were sown in autoclaved commercial soil (SM-2, Ibigawa Industry Co. Ltd. Japan) in plastic containers (31.9 cm × 26.4 cm × 15.3 cm) and fertilized using slow-release granular fertilizer (Long Total 70-day type; N: P: K = 13: 9: 11, JCAM Agri. Co. Ltd., Japan). Eight weeks after sowing, water extracts of the four selected herb shoots were applied (100ml/plant) to the rhizospheric soil of the asparagus seedlings.

Fusarium oxysporum f. sp. *asparagi* (MAFF305556) were cultured on potato-dextrose agar medium and incubated in dark conditions at 25°C for 2 weeks to facilitate sporulation. The conidia were harvested in potato sucrose liquid media and incubated in dark conditions at 25°C for 7 days. The conidial suspension was then sieved and the concentration was adjusted to 10^6

conidia/ml. The conidial suspension was inoculated in the rhizospheric soil (50 ml/plant) immediately following herb extract treatment. Forty plants per plot with three replicates were grown in a greenhouse at 30/24 ± 4°C day/night temperature with 12-13-h photoperiods (750-1000 µmol/m^2/s) and 75%-85% relative humidity. Four weeks after Foa inoculation, the symptoms of Fusarium root rot were assessed on the basis of percentage of diseased roots using 5 scales: 1, <20%; 2, 20–40%; 3, 40–60%; 4, 60–80%; and 5, 80–100%. The disease index was calculated using the following formula:

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

Four weeks after Foa inoculation, the rhizospheric soil was collected for analysis of Fusarium populations. Each soil sample (1 g) was diluted to 10^{-3} with distilled water. Komada medium (Table 1), which is selective for *Fusarium oxysporum* (Komada 1975) was used, and the inoculated media were incubated in dark conditions at 25°C for 5 days to determine the numbers of populations, which were expressed as colony forming units (CFUs).

Evaluation of Rosmarinic acid and caffeic acid for antifungal effect against Foa: Rosmarinic acid and caffeic acid are the most commonly found phenolic acids in *Lamiaceae* herbs and was reported to act as defense compound (Nascimento et al., 2000). As such, these two compounds were evaluated to find out if they have any suppresive activity against Foa *in vitro*. Two milligrams of rosmarinic acid and caffeic acid were separately dissolved in 40 µl of ethanol, and 960 µl of distilled water was added to each of the solutions. Foa, purely cultured in PDA medium, was mixed with Czapek-Dox liquid medium (Czapek 1902; Dox 1910) (Table 2) and incubated in a growth chamber (25°C; in dark conditions) for 2 weeks. In total, 10 ml of the prepared solutions were separately added to freshly prepared Czapek-Dox liquid medium, and in the case of 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 aforementioned Foa conidial suspension (10^6 conidia/ml) was added to each of the Czapek-Dox liquid media containing the different solutions and incubated for 3-5 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 Foa populations in the liquid media were enumerated.

Statistical analysis: Mean values were analyzed by Tukey's multiple range test for dry weight (4 weeks after pathogen inoculation), disease index, colony forming unit and antifungal effect of rosmarinic acid, caffeic acid at $P < 0.05$. All the analysis was conducted using XLSTAT pro statistical analysis software (Addinsoft, New York).

Results

In the bioassay, four weeks after Foa inoculation, the dry weight of shoots and roots of asparagus plants treated with herb extracts became significantly higher ($P < 0.05$) than those of control plants (Fig. 27). Among the different herb extracts, treatment with lemon balm gave the highest root dry weight, although in case of shoot dry weight, no significant difference was observed.

A comparative presentation between healthy and lesioned roots of asparagus is presented in fig. 28. In control plants, the incidence of Fusarium root rot reached 100% with 70% plants exhibiting severity level 5 (Fig. 29A). Conversely, disease incidence in herb treated plants were lower than those of control. Although plants under oregano and hyssop extract treatment had reached severity level 5, the percentages were considerably lower (10.53% and 10%, respectively) compared to control. No plants under the treatment of sage and lemon balm extract showed severity level 5. The disease indices also significantly decreased ($P < 0.05$) in herb extract treated plants compared with the control (Fig. 29B). The lowest was observed under lemon balm treatment to which sage showed statistical similarity.

Application of herb extracts seemed to have a considerable suppressive effect on the total CFUs of Foa in the rhizospheric soil of asparagus plants (Fig. 30). CFUs in the soil of plants treated with lemon balm extract were lowest (0.99×10^5) followed by treatment with sage (1.42×10^5), whereas in control, they were as high as 3.61×10^5.

Evaluation of rosmarinic acid and caffeic acid as the possible antifungal properties in the herbs showed promising results (Fig. 31). The Foa populations in the media containing rosmarinic acid and caffeic acid were considerably lower (84.67×10^4 and 102.67×10^4 CFU/ml, respectively) than those in the control (168×10^4 CFU/ml). In addition, the ethanol

solution used to estimate the effect of ethanol on fungal population exhibited no significant differences compared with that of control.

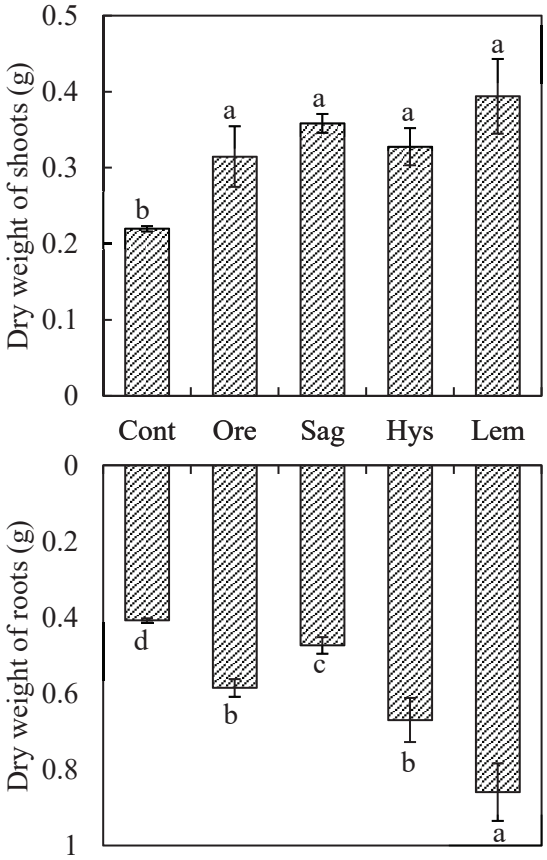


Fig. 27. Dry weight of asparagus in herb extract added soil 4 weeks after Foa inoculation. Here, Cont, control; Ore, oregano; Sag, sage; Hys, hyssop; Lem, lemon balm. Columns denoted by different lettering indicate significant difference according to Tukey's test ($P < 0.05$).

A

B

Fig. 28. Comparison between healthy and lesioned roots of asparagus plants. Here, A, healthy plant's roots; B, lesioned plant's roots.

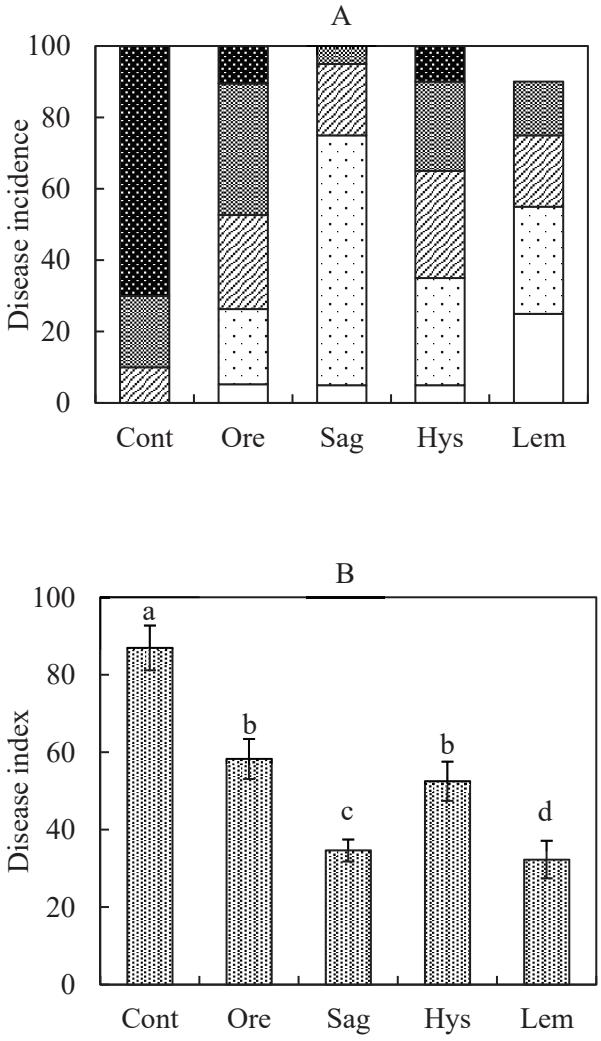


Fig. 29. Inhibitory effect of herb extract on disease incidence (A) and indices (B) in asparagus plants 4 weeks after inoculation. Here, Cont, control; Ore, oregano; Sag, sage; Hys, hyssop; Lem, lemon balm. Columns denoted by different letters indicate significant differences according to Tukey's test ($P < 0.05$). □, < 20%; ⊡, 20–40%; ▨, 40–60%; ▩, 60–80%; ■, 80–100%.

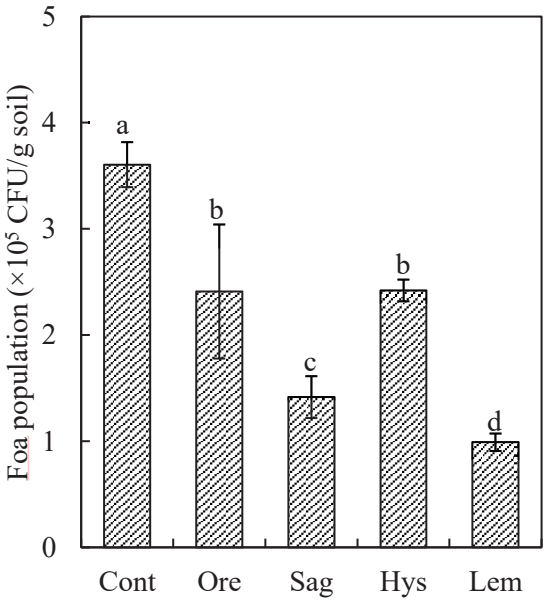


Fig. 30. Influence of herb extracts on Foa population in rhizospheric soil. Here, Cont, control; Ore, oregano; Sag, sage; Hys, hyssop; Lem, lemon balm. Columns denoted by different letters indicate significant difference according to Tukey's test ($P < 0.05$).

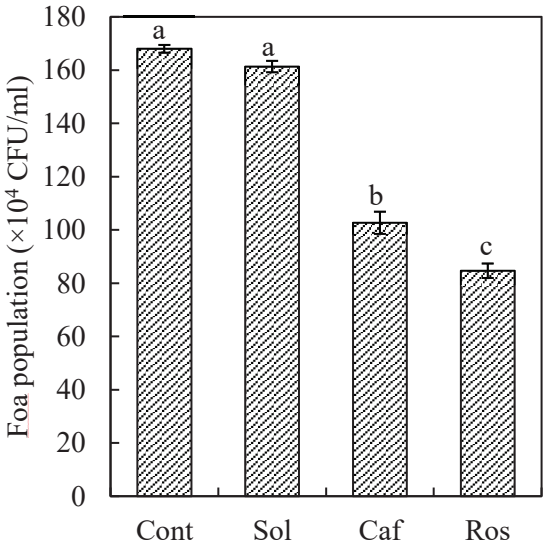


Fig. 31. Influence of chemical compounds on Foa propagation *in vitro*. Here, Cont, control; Sol, solvent (distilled water: ethanol=24:1, v/v); Caf, caffeic acid (200ppm); Ros, rosmarinic acid (200ppm). Columns denoted by different letters indicate significant difference according to Tukey's test ($P < 0.05$).

Discussion

With 230 genus and 7100 species worldwide (Harley et al. 2004), *Lamiaceae* is one of the largest herb family that is of high importance for culinary, medicinal and cosmetic industries. For traditional diet as well as medicine, many *Lamiaceae* herbs and their extracts or tinctures are extensively used (Barros et al. 2011; Carrió and Vallès 2012). A large number of polyphenolic compounds are present in *Lamiaceae* herbs that have a variety of physiological and ecological effect resulting in resistance towards bacterial, fungal and viral infections (Bais et al. 2002). However, such activities are attributed to the volatile EOs present in the herbs (Quintanilla et al. 2002; Vardar et al. 2003; Soylu et al. 2010) and are mainly concentrated on agri-foods and medicinal use (Mamadalieva et al. 2017). In addition, most of the 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 the bioassay, disease incidence and indices in asparagus plants treated with herb extracts were considerably lower than those in control. In additon, a suppressive effect on Fusarium population were observed in rhizospheric soils, indicating fungistatic effects of the herb extracts on the pathogen. Based on the findings, it can be stated that the secondary metabolites present in the water extract of oregano, sage and lemon balm shoots have the potential to suppress Fusarium root rot in asparagus plants. In relation to their *in vitro* assay presented in chapter 1, it can be safely assumed that the contents of the extracts have potential to directly affect the pathogen. Interestingly, hyssop extract also showed similar disease reduction contrary to its *in vitro* evaluation observed previously. The possible explanation of this result could be regulation of the oxidative burst occurring in the plants in diseased condition by the natural antioxidants from the herb extracts. The antioxidants present in the herb extracts may have prevented the cell damage due to high ROS produced by the plants as defense mechanism

against the pathogen. Another way of interpreting this phenomenon is the resistance induction in the crop plant by antioxidants present in the extract. This resistance inducing behavior could be the result of activation of defense mechanism of the crop plant through production of phytoalexins (Huston and Smith, 1980). It was reported that application of aqueous extract of oregano induced 6 times more production of phytoalexins in sorghum plants compared to control (Colpas et al. 2009). A chemical compound will be considered an activator of the systemic acquired resistance (SAR) if it induces resistance to the same spectrum of pathogens and the expression of the same biochemical markers as in the biological model and, in addition, has no direct antimicrobial activity (Kessmann et al. 1994). Here, hyssop extract although did not show antifungal activity in *in vitro* evaluation, may have acted as an elicitor of SAR in the asparagus plants resulting disease free condition. The contrasting result of *in vitro* and *in vivo* further confirms the necessity of evaluation through bioassay as stated by Benner (1993). The bioassay showed the potential multifaceted effect of hyssop extract which was not visible in *in vitro* evaluation, opening up future research question. No phytotoxic effect was observed on the plants treated with water extracts of herbs in the concentration used. This showed the safety of using water extracts for plant treatment unlike EOs which were reported to sometimes having phytotoxic effect (Letessire et al. 2001). The disease suppression consequently led to better growth of asparagus plants, as observed in the form of increased dry weight in both shoots and roots.

Rosmarinic acid and caffeic acids are the most abundantly found phenolics in *Lamiaceae* herbs, although their content vary in different genus as well as in species (Zgórka and Głowniak 2001). These phenolic acids act as both an antioxidants as well as defense compounds (Nascimento et al. 2000; Ibanez et al. 2003; Capecka et al. 2005). It has been also reported that, rosmarinic acid and caffeic acids are water soluble (Sökmen et al. 2004; Widmer et al. 2006). Their presence in water extract were also confirmed by LC-MS/MS analysis in a previous

experiment (Ahmad and Matsubara, 2020a). According to Bais et al. (2002) the antifungal activities of these polyphenolic compounds are exerted through the breakage of intersepta in the mycelia of fungi and cell surface damage through pilferage. Such specific activity against microorganisms makes them potent antimicrobial agents. The results of the current study further confirm the antifungal potential of rosmarinic acid and caffeic acid. In the study, the fungal populations treated with rosmarinic acid and caffeic acid were considerably lower than those in the control *in vitro*. The result is consistent with the decreased fungal populations in rhizospheric soil of asparagus plants in the bioassay of the current study. Therefore, the presence of rosmarinic acid and caffeic acid in the water extract of the herbs plays a key role in exerting the characteristic antifungal effects described in the discussion. Conversely, the methanolic/ethanolic extracts and EOs of herbs have the risk of rapid evaporation from the surfaces on which they are applied, which could reduce the effective concentration of the active compound and enable the disease-causing organism to resume growth (Letessire et al. 2001). However, in the current study, the antifungal effect of the water extract was observed even 1 month after application as demonstrated by the decrease in Foa populations in the rhizospheric soil. So, this propose a sustainable and environment friendly approach of controling fusarium root rot in asparagus to tackle the serious problem of asparagus decline.

To conclude, the current investigation showed that water extracts of oregano, sage, hyssop and lemon balm has direct antifugal effect against Foa and can suppress the Fusarium root rot in asparagus *in vivo*. The presence of secondary compounds like rosmarinic acid and caffeic acid was considred responsible for such activity and might act in synergy when applied as part of herb extract. These confirmed the potential use of *Lamiaceae* herbs to control diseases of a broad spectrum of crops. The findings also expressed evidence of potential multifacetaded way herb extracts could exert such effect like antioxidative defence and inducing disease resistance. The next part of the chapter 2 will focus on the ability of herb extract particularly sage water

extract on suppressing fusarium wilt in cyclamen to evaluate presence of similar disease control.

CHAPTER 2-4

Suppression of Fusarium wilt in cyclamen by using sage water extract and identification of antifungal metabolites

Introduction

Cyclamen is a genus of herbaceous perennials with bright, colorful flowers that are widely grown as garden and house plants (Yesson et al. 2009). Among them, *Cyclamen persicum* (Mill.) is the most popular species used for commercial cultivation worldwide (Ishizaka et al. 2002; Elmer and Daughtrey 2016). However, cyclamen production has decreased abruptly because of several biotic and abiotic stresses (Wright et al. 2006; Rivera et al. 2009; Grover et al. 2011). Among all the biotic factors, Fusarium wilt of cyclamen caused by *Fusarium oxysporum* f. sp. *cyclaminis* is a highly destructive disease that prevents good-quality cyclamen production and can cause up to 90% loss (Elmer 2002). The pathogen can cause the sudden death of plants at all stages of plant development, with symptoms such as stunting, chlorosis, and unilateral wilt that causes the collapse of the entire plant (Lori et al. 2012). The disease is very difficult to control because of the lack of adequate measures. No commercially acceptable resistant germplasm is available for cyclamen production (Orlicz-Luthardt 1998), and the chemical fungicides used for disease control have poor curative properties (Elmer and McGovern 2004). Moreover, repeated applications of broad-spectrum or persistent fungicides may result in soil contamination, fungicide resistance, or harmful effects on non-target organisms (Someya et al. 2000). Therefore, the search for alternative and environment friendly approaches for anthracnose control has become a challenge for cyclamen production.

Herbs that belong to the family *Lamiaceae* contain several phenolic compounds, terpenoids, and glucosides as secondary metabolites with antimicrobial and antioxidant activities (Martino et al. 2009; Stanojevic et al. 2010; Weerakkody et al. 2011). The antimicrobial and preservative activities of essential oils (EOs) in the herbs have been well documented, primarily in 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). Quintanilla et al. (2002) showed that the

EOs of herbs such as thyme, oregano, lemon balm, and peppermint inhibited the growth of *Phytophthora infestans* in an *in vitro* plate assay. In addition, the EOs of lavender and rosemary were found to suppress the *in vitro* growth of *Botrytis cinerea* (Soylu et al. 2010). The closed environment in the *in vitro* experiments enables the volatile active compounds of the EOs to accumulate and inhibit fungal growth. However, under field conditions, these volatile EOs diffuse away spontaneously, which reduces the effective concentration for antifungal effects (Letessier et al. 2001) and makes the use of EOs as a disease control measure impractical for field conditions. In addition, EOs have phytotoxic effects on crops after foliar application at high concentrations (Letessier et al. 2001). A viable environment-friendly alternative could be the use of water extracts containing non-volatile secondary metabolites. Water extract preparation is a relatively easy and inexpensive process when compared with that of EOs. In addition, as the extracts are non-volatile, they remain effective for longer periods than EOs. However, besides *in vitro* analysis, bioassays of such extracts through application on 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). Therefore, this study was conducted to evaluate the antifungal activities of sage water extracts against Fusarium wilt in cyclamen through bioassay. Furthermore, the important secondary metabolites present in the water extract were identified, and their antifungal potential was evaluated.

Materials and Methods

Growing of sage plants: Seeds of sage (*Salvia officinalis* L) were sown in plastic containers (31.9 × 26.4 × 15.3 cm) containing commercial soil (Supermix A; Sakata Co. Ltd., Japan) and grown in a greenhouse. After eight weeks, the plants were uprooted, and the shoots were cryopreserved using liquid nitrogen.

Bioassay of the sage extract for Fusarium wilt control in cyclamen: For the bioassay, frozen sage samples were ground in distilled water by using a mixer, and the herbal extract concentration was maintained at 20% (w/v). The extract was filtered and used as the herb water extract.

Cyclamen (*Cyclamen persicum* 'Pastel') seeds were sown in trays with sterile peat moss, perlite, and sand mix (1:1:1); three months later, the seedlings were replanted in plastic pots (10.5 cm in diameter, 0.5 L) containing autoclaved commercial potting media (peat moss mixed) and fertilized using a slow-release granular fertilizer (Long total 70-day type; N:P:K = 13:9:11; JCAM AGRI. Co. Ltd., Japan). Ten weeks after transplantation, sage water extract (20%, w/v) was applied (50 ml/plant) two times before pathogen inoculation. In the control treatment, distilled water was used (50 ml/plant). *Fusarium oxysporum* f. sp. *cyclaminis* strain MAFF 712100 was cultivated on PDA and incubated in the dark at 25°C for two weeks to facilitate sporulation. The conidia were harvested in potato sucrose liquid media and incubated in the dark at 25°C for seven days. The conidial suspension was then sieved, and the concentration was adjusted to 10^6 conidia/ml. The rhizospheric soil of each cyclamen plant was inoculated with the conidial suspension (50 ml/plant) immediately after the second application of the sage extract and distilled water for the herb-treated and control plants, respectively. Ten plants per treatment with three replicates were maintained in a growth chamber at 22 ± 2 °C, 12 h photoperiod (750–1000 μmol/m^2/s), and 60–70% relative humidity. Six weeks after inoculation, 10 plants were selected from each treatment, and the symptoms of Fusarium wilt were assessed on the basis of the percentage of diseased shoots by using five scales: 1, < 20%; 2, 20–40%; 3, 40–60%; 4, 60–80%; and 5, 80–100%; three scales were used for the roots: 1, partly diseased; 2, half-diseased; and 3, all diseased. The disease index was calculated using the following formula:

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

Ten plants from each treatment were separated into shoots and roots and dried using a constant temperature drier (ETTAS EO-600B, AS ONE Co., Japan) at 80 °C for 2 days. Then, dry weights of the shoots and roots were measured.

Six weeks after the inoculation, the roots were sampled from all treatments. One gram of root sample was diluted to 10^{-4} with distilled water. Komada medium (Table 1), which is selective for *F. oxysporum* (Komada 1975), was used. The inoculated medium was incubated in the dark at 25 °C for five days to determine the population numbers, and these were expressed as colony forming units (CFUs).

Analysis of sage water extract using UPLC-MS: From the cryopreserved samples of five sage plants, 0.6 g of sage shoots was pulverized in a mortar with liquid nitrogen to obtain a fine powder, and the powder was mixed with 3 ml of ultrapure water to prepare a sample extract solution (20%, w/v). The sample solution was then centrifuged (13,000 rpm, 4 °C, and 15 min), and the supernatant was passed through a sterile filter (0.45 μm; Advantech Co. Ltd., Japan). The sample was centrifuged (13,000 rpm, 4 °C, and 15 min) using Nanosep 10K (Pall Corporation, Tokyo, Japan) to remove the proteins from the extract.

The samples were analyzed using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-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 thermostation at 25 °C was used for the analysis. The mobile phase was 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. A mass spectrometer (Xevo Q Tof MS; Waters

Corporation, Milford, USA) was used to analyze the mass range of electrospray ionization in the negative mode at 50–1000 m/z; MS/MS collision was performed at 30 V. A mass chromatogram of the m/z value of each component in the extract was prepared from the results obtained using retention time.

Evaluation of the identified chemicals for antifungal effects on *F. oxysporum* f. sp. *Cyclaminis*: Two milligrams of rosmarinic acid and two mg of caffeic acid (identified in the water extract of sage shoots) were separately dissolved in 40 µl of ethanol, and 960 µl of distilled water was added to each of the three solutions. *Fusarium oxysporum* f. sp. *cyclaminis*, purely cultured in PDA, was mixed with Czapek-Dox broth (Czapek 1902; Dox 1910) (Table 2) and incubated in a growth chamber (25 °C, in the dark) for two weeks. In total, 10 ml of the prepared solutions were separately added to freshly prepared Czapek-Dox broth; for the control, distilled water was added. To factor out the effect of ethanol used for the solution preparation, a simple ethanol solution (ethanol:distilled water = 1:24, v/v) was also evaluated for comparison. The prepared conidial suspension (10^6 conidia/ml) was added to Czapek-Dox broths containing the different solutions and incubated for five days in a growth chamber (25 °C, in the dark). At the end of the incubation, the density of conidia was investigated using a hemocytometer. The average values were calculated from nine replicates, and the propagation index for *F. oxysporum* f. sp. *cyclaminis* was recorded.

Statistical analysis: The mean values were analyzed with Student's *t*-test for dry weight, disease index and CFU in roots whereas antifungal effects of rosmarinic acid and caffeic acid were evaluated using Tukey's multiple range test at $P < 0.05$. All the analyses were conducted using XLSTAT 2012 pro statistical analysis software (Addinsoft, New York).

Results

In the bioassay, the dry weights of the shoots and roots of cyclamen treated with sage extract showed a significant increase six weeks after *F. oxysporum* f. sp. *cyclaminis* inoculation (Fig. 32). The incidence of Fusarium wilt in the shoots was more than 80% in the control plants, with 16% plants scoring a severity level of 5; however, the incidence of Fusarium wilt was nil in the plants treated with sage extract (Fig. 33A, 34). In the case of roots, disease incidence was far worse in the control plants, with 100% plants being completely diseased (Fig. 33A). Conversely, although the roots treated with sage extract exhibited an incidence of 100%, only 33% plants were completely diseased. Therefore, the disease index significantly decreased in the plants treated with sage extract with respect to both shoots (0 vs. 1.8) and roots (72.2 vs. 100) (Fig. 33B).

The application of sage extract also had a considerable suppressive effect on the total CFUs of *F. oxysporum* f. sp. *cyclaminis* in the cyclamen plant roots (Fig. 35). CFUs in the roots of the plants treated with sage extract were as low as 2.7×10^3, whereas they were as high as 8.5×10^3 in the control.

The sage water extract was analyzed using UPLC-MS (Fig. 36A, B). The chromatogram showed that the most promising compounds had retention times ranging from 9.95 min to 17.01 min. Two potentially important peaks were observed at 9.95 min and 13.87 min. The presence of pseudo-molecular ions [M_H] at approximately 175 m/z and 345 m/z was observed at the corresponding retention times. Cross-referencing the values in MassBank (https://massbank.eu/MassBank/) revealed that the compounds were caffeic acid and rosmarinic acid, respectively.

Caffeic acid and rosmarinic acid were evaluated for antifungal effects on *F. oxysporum* f. sp. *cyclaminis in vitro* (Fig. 37). The propagation indexes for *F. oxysporum* f. sp. *cyclaminis* in

the media containing caffeic acid and rosmarinic acid were considerably low, especially in the case of rosmarinic acid (less than 50). In addition, the effects of the ethanol solution (used for compound solution preparation) on fungal propagation were not significant, as observed from its negligible suppression of *F. oxysporum* f. sp. *cyclaminis in vitro* (97.6).

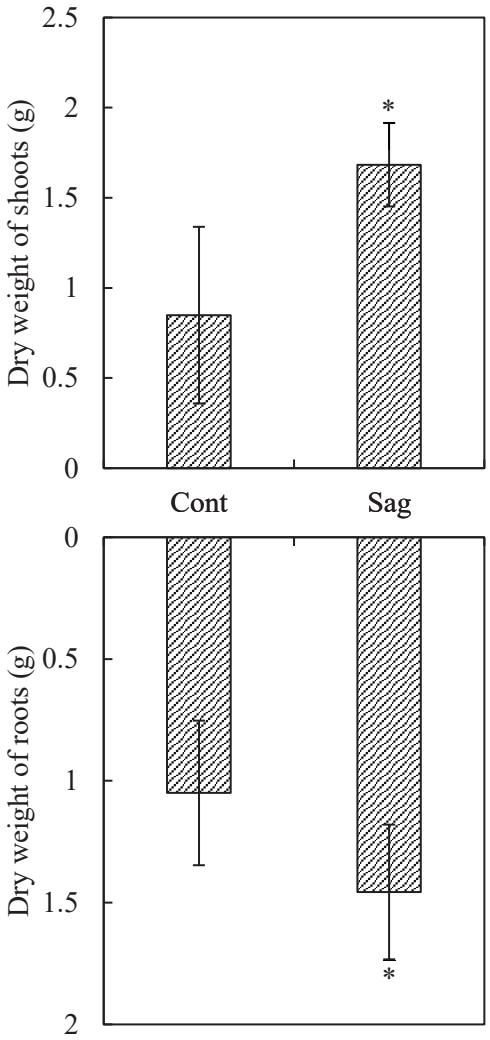


Fig. 32. Dry weight of cyclamen shoots and roots in sage extract-added soil six weeks after *F. oxysporum* f. sp. *cyclaminis* inoculation. Cont, control; Sag, sage. *, significant difference according to *t*-test ($P < 0.05$).

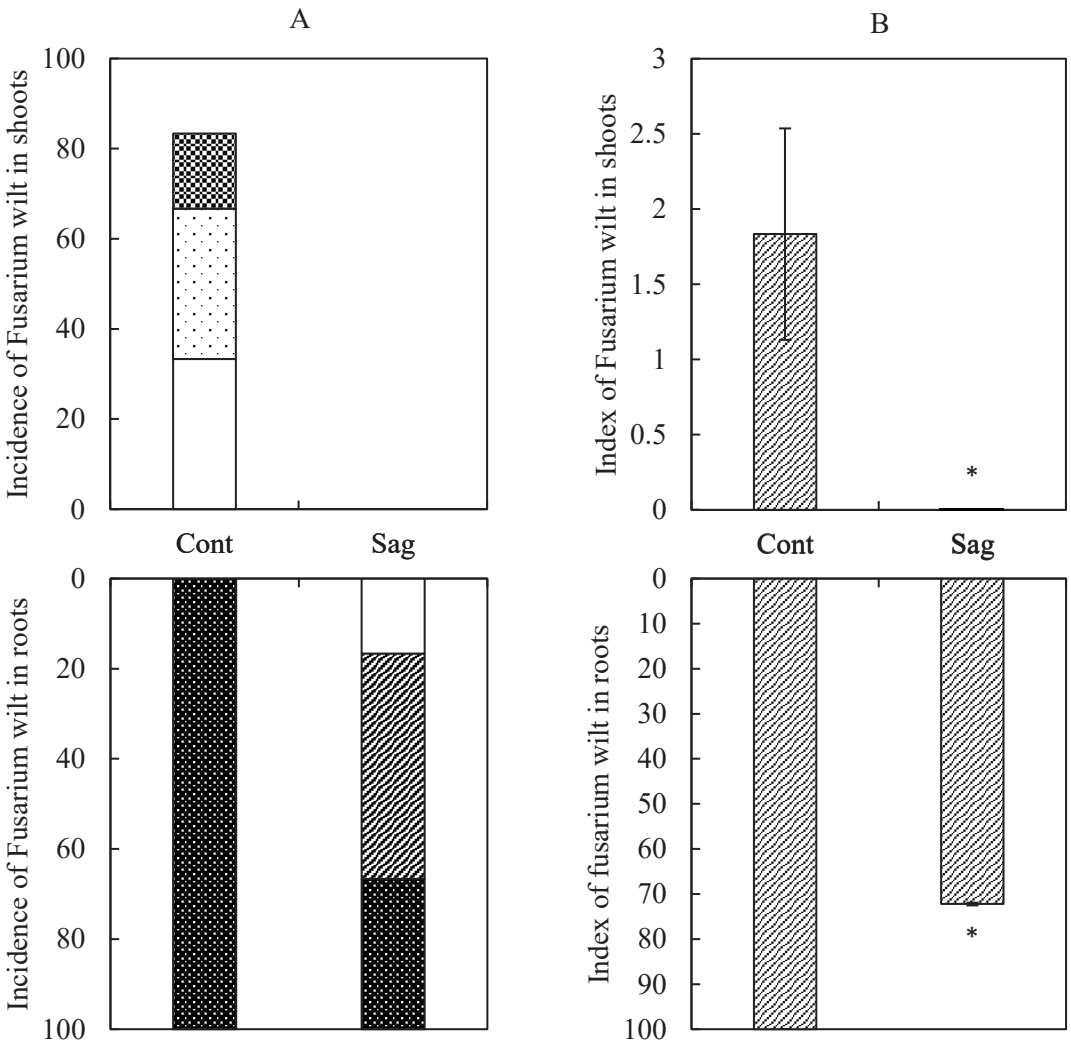


Fig. 33. Inhibitory effect of sage extract on disease incidence (A) and indices (B) in cyclamen plants 4 weeks after inoculation. Here, Cont, control; Sag, sage. Columns denoted by different letters indicate significant differences according to Tukey's test ($P < 0.05$). □,< 20%; ⊡, 20–40%; ⊡, 40–60%; ▨, 60–80%; ■, 80–100%.

Cont

Sag

Fig. 34. Effect of sage extracts on fusarium wilt of cyclamen. Here, Cont, control plants; Sag, Sage.

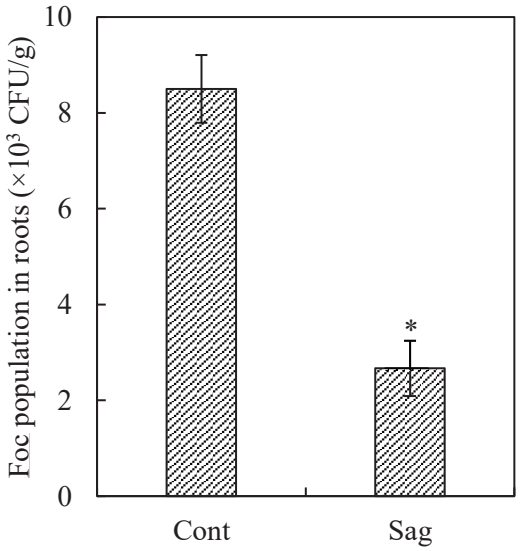
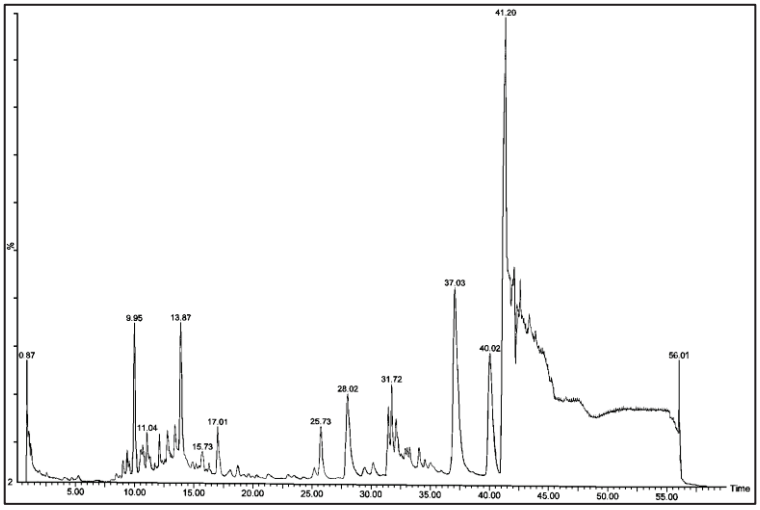


Fig. 35. Influence of sage extract on *F. oxysporum* f. sp. *cyclaminis* population in cyclamen roots. Cont, control; Sag, sage. *, significant difference according to *t*-test ($P < 0.05$).

A



B

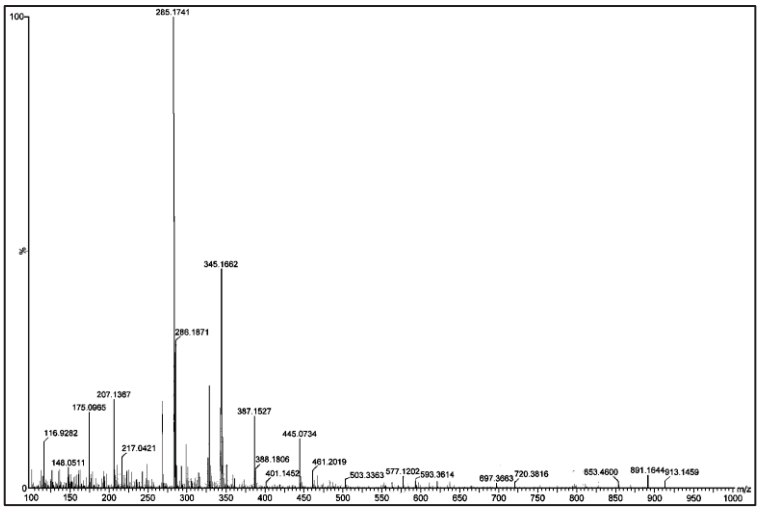


Fig. 36. Chromatogram (A) and mass spectrum (B) of sage water extract derived by UPLC-MS.

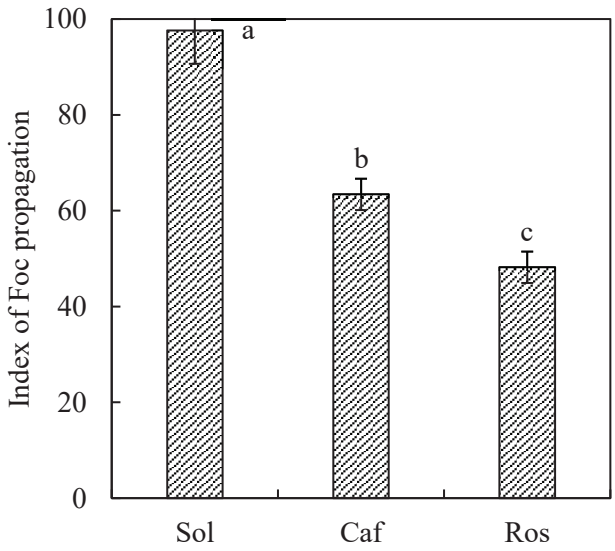


Fig. 37. Influence of chemical compounds on *F. oxysporum* f. sp. *cyclaminis* propagation *in vitro*. Sol, solvent (distilled water: ethanol=24:1, v/v); Caf, caffeic acid (200ppm); Ros, rosmarinic acid (200ppm). Columns denoted by different letters indicate significant difference according to Tukey's test ($P < 0.05$).

Discussion

In this study, sage was selected for the bioassay evaluation against Fusarium wilt of cyclamen. From the result of *in vitro* assay against *F. oxysporum* f. sp. *cyclaminis* presented in chapter 1, among the ten herbs, the highest suppression was observed from lemon balm extract. However, the suppressive ability of lemon balm extract against Fusarium species had already been confirmed through bioassay against Fusarium wilt in strawberry. As sage expressed the second highest suppression among the others, it was evaluated to see whether it would express a similar suppression in bioassay against Foc and could be presented as a substitute to lemon balm.

In the bioassay, the incidence of Fusarium wilt in both shoots and roots was considerably lower in the plants treated with sage than in the control. In addition, a suppressive effect on the *Fusarium* population was observed in the plant roots treated with sage, indicating the fungistatic and fungicidal effects of the sage extract on the pathogen. On the basis of these results, secondary metabolites present in the water extract of sage shoots have the potential to suppress Fusarium wilt in cyclamen. In addition, the disease suppression led to better growth of cyclamen, as shown by the increased dry weights of both shoots and roots.

UPLC-MS analysis of sage water extract yielded two potentially important peaks, and their corresponding m/z values were cross-referenced with MassBank; the two compounds were identified as caffeic acid and rosmarinic acid. A diverse number of caffeic acid oligomers have been reported to be present in sage extracts, and they act as scavengers of 1,1-diphenyl-2-picrylhydrazyl and super oxide anions (Bors et al. 2004; Pavić et al. 2019). The presence of rosmarinic acid in sage extract has also been reported in several studies (Bandoniene et al. 2005; Oliveira and Oliveira 2013), and it plays an important role in the antioxidative and antimicrobial effects of the extract. The presence of rosmarinic acid and caffeic acid in the sage

extract hints towards to fact that they are possibly principal components for disease suppression in the extract; therefore, to confirm this, we performed the *in vitro* antifungal assay.

The two identified compounds showed considerable suppression of *F. oxysporum* f. sp. *cyclaminis* in the *in vitro* antifungal assay. The result is consistent with the decreased fungal populations in the cyclamen roots in the bioassay. Phenolic acids such as rosmarinic acid and caffeic acid act as phytoanticipins in plants (Dixon 2001). Bais et al. (2002) reported that the antifungal activity of rosmarinic acid is exerted through breakage of the interseptum of fungal mycelia and damage to the fungal cell surface by pilferage. Such specific activities of rosmarinic acid against microorganisms make it a potent and novel antimicrobial agent. Caffeic acid is an elicitor of the plant defense response, and its antifungal effects have been confirmed by many studies (Harrison et al. 2003; Santiago et al. 2010). Phenolic acids like rosmarinic acid and caffeic acid have anti-biofilm formation and cell membrane disruption abilities, which play an important role in their antimicrobial effects (Sung and Lee 2010). Therefore, the presence of caffeic acid and rosmarinic acid in sage extract could be responsible for the antifungal effects of the extract. Furthermore, the compounds could have worked synergistically in suppressing the pathogen when applied via the herb water extract, which suggests the potential of using water extracts as antimicrobial agents. Moreover, methanolic/ethanolic extracts and EOs of herbs rapidly evaporate 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 even one month after application, as demonstrated by the decrease in *F. oxysporum* f. sp. *cyclaminis* populations in the cyclamen roots. The extract preparation procedure in this study was simple, inexpensive, and sustainable, and the extract concentrations were comparable to those obtained using other extraction methods.

In this study, the direct effects of the antifungal properties of sage extract on *F. oxysporum* f. sp. *cyclaminis* and subsequent disease suppression were observed. Beside this, 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 higher antioxidant activity in the presence of herb extracts. Further analyses are required to confirm these possibilities and determine the mechanism underlying disease suppression in herb-treated plants.

Chapter 2-Conclusion

From the findings of the experiments presented in this chapter, it can be concluded that, *Lamiaceae* herbs water extracts especially oregano, sage, hyssop and lemon balm possessed the ability to suppress phytopathogens that causes diseases to several important horticultural crops. Among these 4 herbs, water extract of lemon balm was found to be able to suppress a wide range of the phytopathogens in bioassay which showed its potential applicability in practical production system. To identify the components of the herbs responsible for this antifungal activity, the herb extracts were analyzed using UPLC-MS and presence of important secondary metabolites like rosmarinic acid, caffeic acid, luteolin apigenin and protocatechuic acid were found in the herb extracts. These compounds were evaluated through *in vitro* analysis against the phytopathogens and were confirmed to be responsible for the antifungal activity of the herbs extracts. So, the water extracts of the *Lamiaceae* herbs could be used as an alternative and eco-friendly way to suppress several fungal pathogens that damages importance horticultural crops. In this view, improving the quality of the herbs to improve the active compounds presence is necessary to further boost these disease control effect. The next chapter will address this point through evaluation of arbuscular mycorrhizal colonization on growth and quality improvement of herbs. Furthermore, the synergistic effect of arbuscular mycorrhizal fungi and herb extract treatment in inducing disease resistance in horticultural crops will be evaluated using anthracnose of strawberry as a model.

CHAPTER 3-1

Influence of arbuscular mycorrhizal fungi on growth and secondary metabolites in *Lamiaceae* herbs

Introduction

As a reservoir of various natural products used as medicine, nutrition, perfume, food supplements and others, medicinal plants can be considered as a boon from nature to mankind's survival (Mathela and Kumar, 2018). Among the range of medicinal plants, those under *Lamiaceae* family have been the focus of the researchers for their diverse chemical and biological activity. Formerly known as *Labiateae*, the members of this family have long since used as traditional medicine, flavoring agent, fragrance and cosmetics. Oregano, sage, hyssop, lemon balm, basil, mint, rosemary, thyme, perilla, marjoram etc. are some of the well-known plants under *Lamiaceae* for their medicinal and essential oil production (Harley et al., 2004; Stanko et al., 2016). The presence of various biologically active secondary metabolites in these herbs are responsible for their importance in drug discovery as well in cosmetics, foods and pesticides industries (Khaleda-Khodja et al., 2014; Hussein, 2018). Many active compounds that are being used in modern medicinal industries have been derived from these naturally occurring secondary metabolites discovered in these herbs (Sharma and Canoo, 2013; Mathela 2017). Besides the pharmacological uses, the antioxidative and antimicrobial activities of the *Lamiaceae* herbs extracts against plant pathogen *in vitro* have also been reported (Gomes et al., 2014). As such, improvement of the growth and quality of these herbs is important from the aspect of ensuring the wellbeing of mankind.

One of the eco-friendly ways to improve the quality of plants is through application of arbuscular mycorrhizal fungi (AMF) during the cultivation process. AMF association occurs with majority of the territorial plants found worldwide including various medicinal plants (Brundrett, 2009). The association represents a mutualistic relation where the mycorrhiza supplies nutrients and water to the plant and in turn, plant provides carbohydrates necessary for the survival of the mycorrhiza (Smith and Read, 2008). AMF association promotes growth and development in plants through contributing in nutrient cycling, soil structure management

along with improved supply of nutrient to the host plant (Truber and Fernandes, 2014). Besides growth promotion, AMF association has also been reported to increase the production of active compounds and therapeutic substances in medicinal plants (Zeng et al., 2013; Lermen et al., 2017).

The findings of the previous chapter have shown the importance of the *Lamiaceae* herbs secondary metabolites on the aspect of controlling phytopathogen. The findings provided a new eco-friendly disease control approach towards several fungal pathogens that hampers the production of some important horticultural crops. As such improvement of the secondary metabolites content in these herbs through AMF association could potentially improve the effectivity of their water extracts. However, information regarding the impact of AMF inoculation on the change in the content of water-soluble secondary metabolites of *Lamiaceae* herbs is scarce. With this view in mind, the following experiment was conducted to evaluate the AMF association effect on the growth of some important herbs under *Lamiaceae* and subsequent secondary metabolite change in their water extracts.

Materials and Methods

Growing of *Lamiaceae* herbs and AMF inoculation: Seeds of oregano (*Origanum vulgare* L.), sage (*Salvia officinalis* L.), basil (*Ocimum basilicum* L.), hyssop (*Hyssopus officinalis* L.), peppermint (*Mentha piperita* L.) and lemon balm (*Melissa officinalis* L.) (Fig. 38) were sown in plastic containers (31.9 cm × 26.4 cm × 15.3 cm) containing autoclaved commercial soil (Supermix A, Sakata Co. Ltd., Japan). At the time of seed sowing, containers under the AMF treatment were inoculated with 3 g/plant of commercial mycorrhizal fungus inocula (*Gigaspora margarita*, GM; *Glomus fasciculatum*, Gf; obtained from Centralgrass Co. Ltd. Tokyo, Japan and Idemitsuagri Co. Ltd., Tokyo, Japan respectively; spore density unknown). The non-mycorrhizal containers received the same amount autoclaved (121 °C, 1.2 kg/cm^2, 30

min) inocula. Twenty plant/plot were grown in a greenhouse at 30 ± 4/24 ± 4 °C temperature with 12-13 h photoperiods (750-1000 µmol/m2/s) and 60-70% relative humidity.

Evaluation of growth promotion of herbs by AMF: Eight weeks after mycorrhizal inoculation, the herb plants were uprooted and the roots were cleaned carefully under slow flowing tap water in a try to remove soil and prevent loss of fine roots. Six plants from each treatment were separated in to shoots and roots which were then dried using a constant temperature drier (ETTAS 600B) at 80 °C for 2 days. After drying the dry weights of shoots and roots were measured. The rest of the plants were cryopreserved for metabolomic analysis.

Determination of AMF colonization: Lateral roots of the herb plants were preserved with 70% ethanol and stained according to the method of Phillips and Hayman (1970). The roots were washed under tap water to remove the 70% ethanol used for preservation and then autoclaved at 121 °C, 1.2 kg/cm^2 for 15 min. After autoclaving, the roots were immersed in 10% potassium hydroxide solution. Then, the roots were washed with distilled water to remove the potassium hydroxide solution and stained with trypan blue solution (glycerin 50 ml, lactic acid 50 ml and trypan blue 1 g). Thereafter, the stained roots were cut into 0.5 to 1.0 cm sections and placed on a glass slide for observing the colonization using an optical microscope. The percentage of AMF colonization was calculated using approximately 50 samples of 1 cm segments per plant from 10 randomly selected plants.

Determination of secondary metabolites change by AMF in herb water extracts: The samples were selected based on the results of growth promotion observed by dry weight analysis in each herb under AMF treatments and was compared with plants under control treatment respectively. From the cryopreserved samples of five plants, 0.6 g of herb leaves were pulverized separately in a mortar with liquid nitrogen to a fine powder and mixed with 3 ml of ultrapure water to prepare a sample extract solution (20%, w/v) for each. The sample

solutions were then centrifuged at 13,000 rpm for 15 min at 4 °C using Nanosep 10K (Pall Corporation, Tokyo, Japan) to remove proteins in the extracts; the supernatants were filtered through a sterilizing filter (0.45 μm, ADVANTECH Co. Ltd., Japan).

The samples were analyzed 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 of 0.1% formic acid in water (A) and acetonitrile (B) at a flow rate of 0.4 ml/min. The sample injection volume was 10 μl. The gradient profile was as follows: 0–6 min, 95% A; 6–12 min, 75% A; 12–30 min, 65% A; 30–32.5 min, 5% A; and 32.5–35 min, 95% A. The mass range of electrospray ionization was analyzed in negative mode at 50-1000 m/z using a mass spectrometer (Xevo Q Tof MS, Waters Corporation, Milford, USA), and the MS/MS collision was performed at 30 V. A mass chromatogram of the m/z value of each component in the extract was prepared from the measurements obtained using the retention time.

Statistical analysis: Mean values were analyzed by Tukey's multiple range test for dry weights of shoots and roots of herbs and AMF colonization rate at $P < 0.05$. All the analyses were conducted using XLSTAT pro statistical analysis software (Addinsoft, New York).

Lemon balm
(*Melissa officinalis* L.)

Oregano
(*Origanum vulgare* L.)

Hyssop
(*Hyssopus officinalis* L.)

Basil
(*Ocimum basilicum* L.)

Sage
(*Salvia officinalis* L.)

Peppermint
(*Mentha piperita* L.)

Fig. 38. Herb plants used in the experiment.

Results

Herb growth response: The dry weights of shoots and roots of herbs under AMF treatment showed considerable variation compared to control (Fig. 42). Significantly higher dry weight of shoots and roots compared to control were observed in lemon balm under Gf and in oregano and hyssop under GM treatment (Fig. 39, 40). In basil, higher dry weight of shoots was observed in plants under GM treatment compared to control although no significant difference was observed regarding root dry weights (Fig. 40). In case of sage and peppermint, no significant difference in both shoots and roots dry weights were observed in plants under AMF treatment compared to control (Fig. 41).

AMF colonization: Successful colonization of the herb plants were confirmed by microscopic analysis regarding plants under AMF treatment (Fig. 43). Plants under control did not show any colonization. Regarding level of colonization, the herbs under consideration did not show a considerable variation when compared to each other. In regards to the two species used; no significant difference was observed in most of the herbs except in lemon balm where Gf showed a higher level of colonization than GM.

Influence of AMF treatment on secondary metabolites of herb water extracts: The analysis of water extracts of the herbs was conducted using UPLC-MS/MS and represented in the form of chromatograms and spectrum graphs (Fig. 44-49). From the chromatograms, the most promising region of compounds with high peaks was observed between the retention time of 5 to 23 min in case of all the herb extracts. Presence of kaempferol with approximate m/z value to 286 was observed in water extracts of lemon balm, oregano, hyssop and sage under control treatment at the retention times of 18.51 min, 18.42 min, 17.43 min and 17.43 min respectively (confirmed by cross-referencing the data with the mass bank, https://massbank.eu/MassBank/). However, an increased content of the kaempferol was

observed in the herb plants that were under AMF treatment in all the cases confirmed by the higher peak size when compared to control. Besides kaempferol, presence of geniposide (m/z value of approximately 388.36) was also confirmed in oregano and sage (retention time 9.15 min) and their content was also significantly increased under AMF treatment. In lemon balm, presence of luteolin was also confirmed at a retention time of 12.90 min with larger peak from plants under AMF treatment. Apigenin (m/z value of approximately 270.05) was identified in the water extracts of both sage and peppermint under control. Contrary to the increase in apigenin content under AMF treated plot as observed with other identified compounds in sage, peppermint extract showed a decreased content with AMF. However, a completely new compound identified as glucosinolate (m/z value of approximately 437.05) was found in it at a retention time of 13.34 min which was absent in control extracts. Similarly, in basil, decrease in coumaric acid concentration was observed in plants under AMF with the presence a new compound (retention time 13.07 min) identified as caffeic acid (m/z value of approximately 180.04) when compared to control.

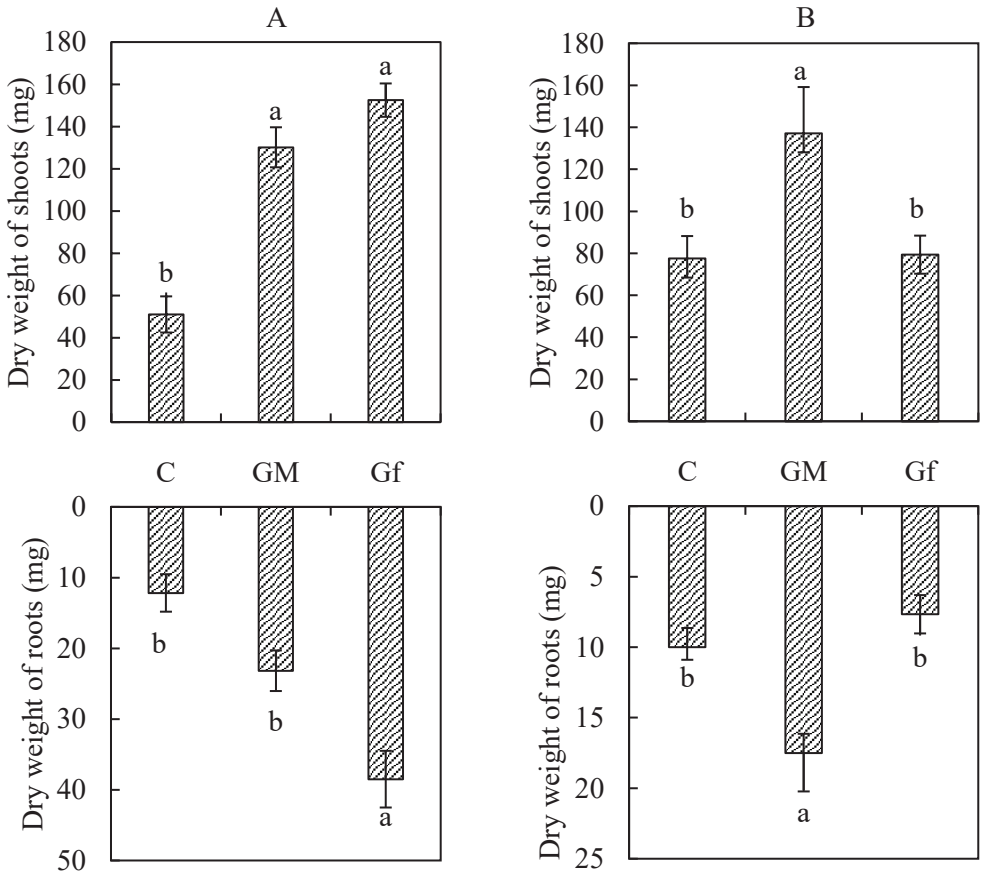


Fig. 39. Dry weight of shoots and roots of lemon balm (A) and oregano (B) under AMF colonization. Here, C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum.* Columns denoted by different letters indicate significant difference according to Tukey's test ($P < 0.05$).

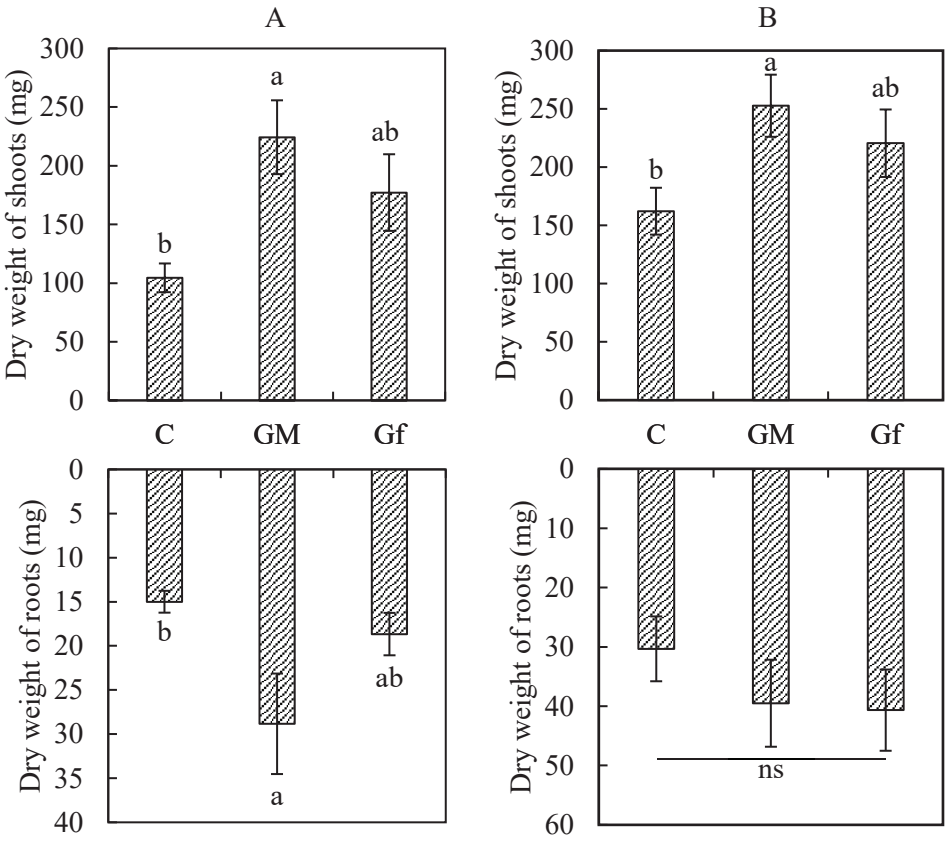


Fig. 40. Dry weight of shoots and roots of hyssop (A) and basil (B) under AMF colonization. Here, C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum.* Columns denoted by different letters indicate significant difference according to Tukey's test ($P < 0.05$).

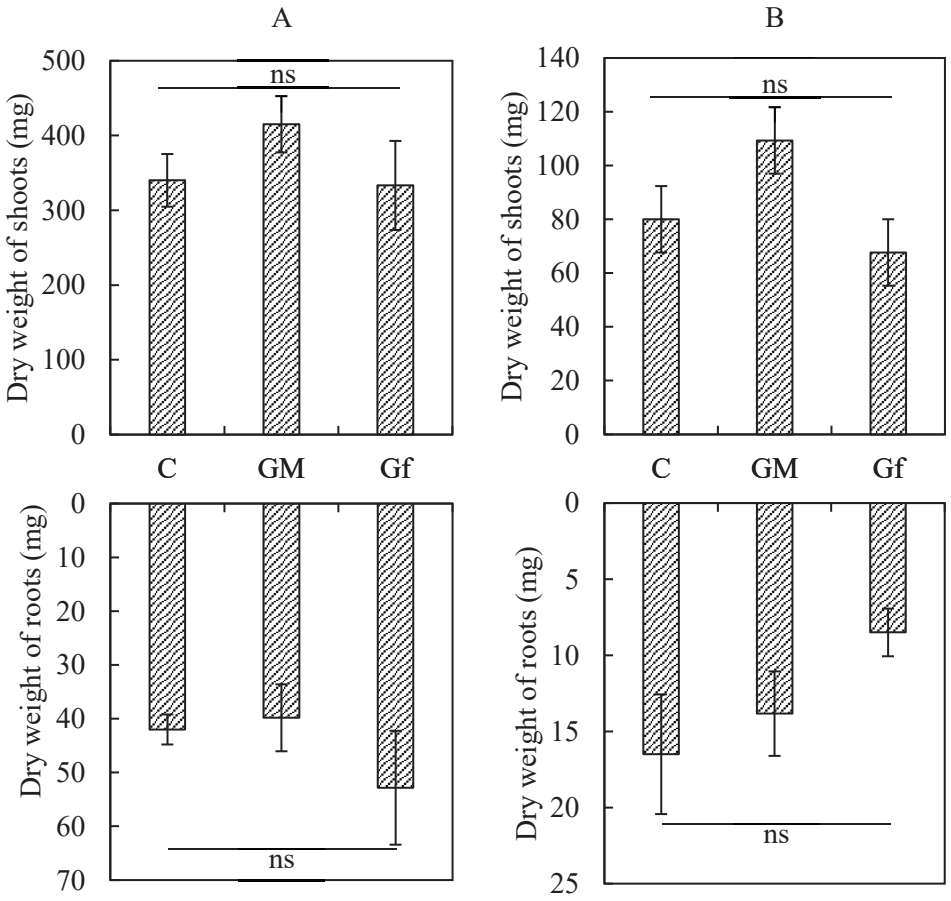


Fig. 41. Dry weight of shoots and roots of sage (A) and peppermint (B) under AMF colonization. Here, C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum.* Columns denoted by different letters indicate significant difference according to Tukey's test ($P < 0.05$).

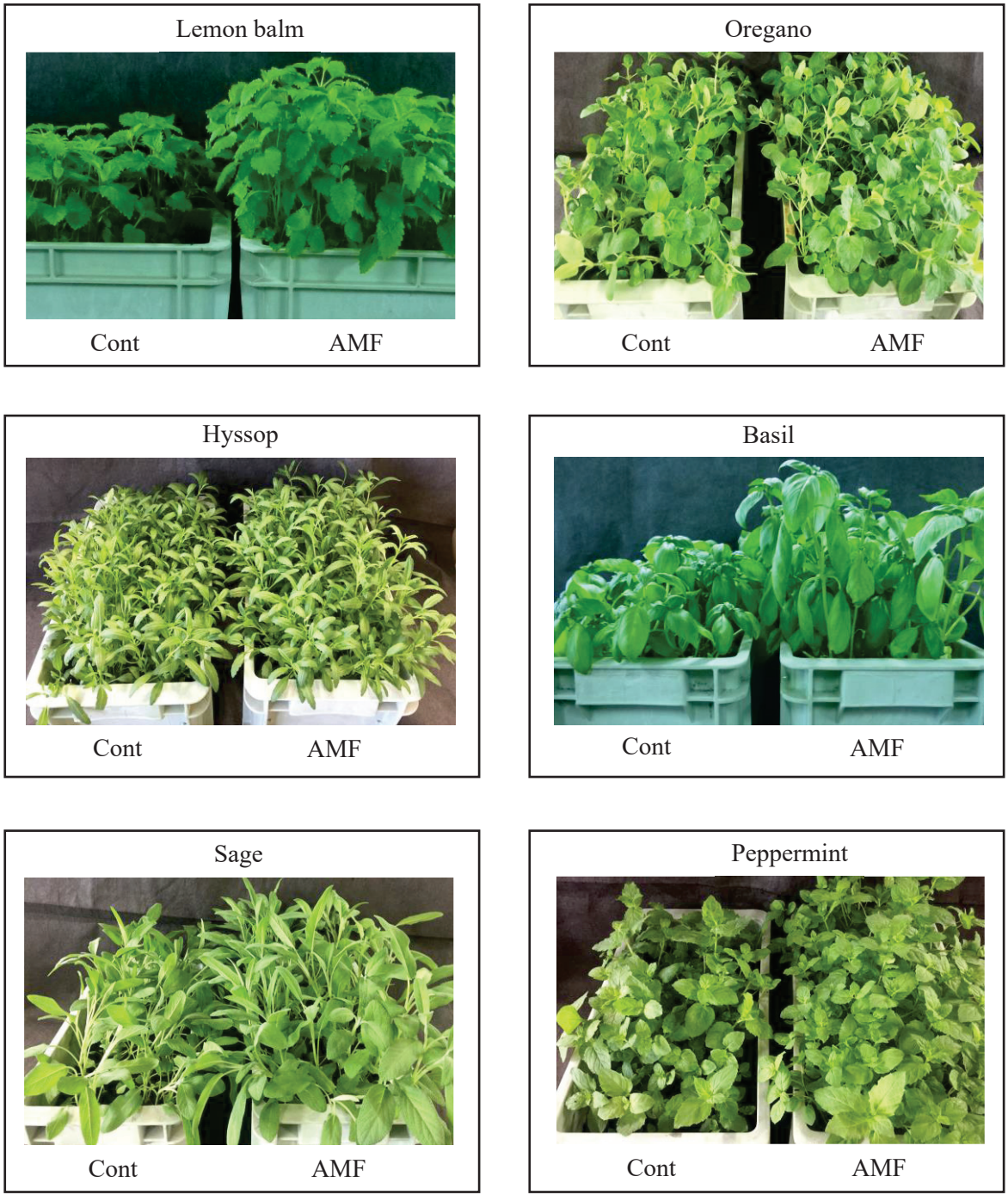


Fig. 42. Influence of AMF on growth of *Lamiaceae* herbs. Here, Cont, control plants; AMF, mycorrhizal plants.

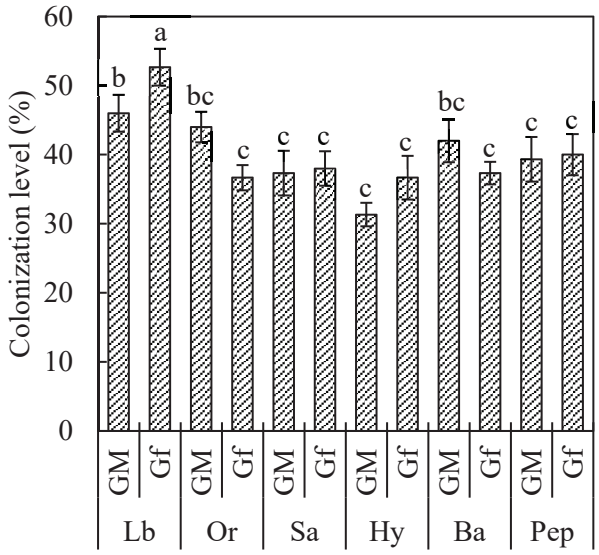
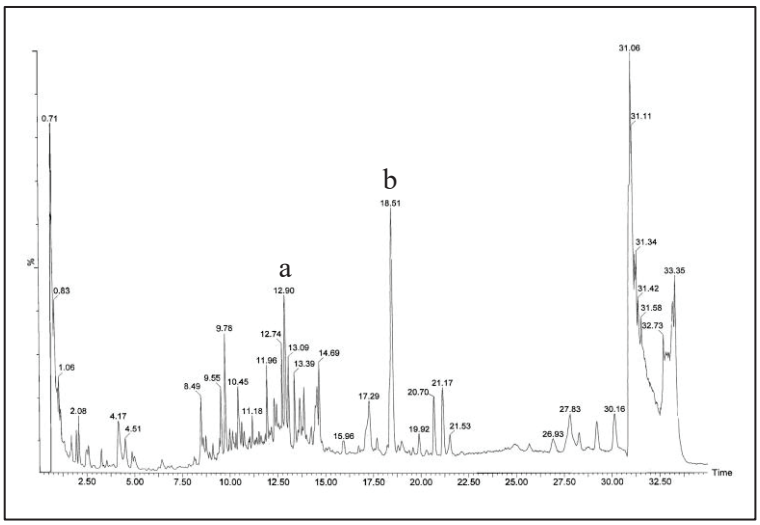


Fig. 43. AMF colonization level in herbs. Here, Lb, lemon balm; Or, oregano; Sa, sage; Hy, hyssop; Ba, basil; Pep, peppermint; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum.* Columns denoted by different letters indicate significant difference according to Tukey's test ($P < 0.05$).



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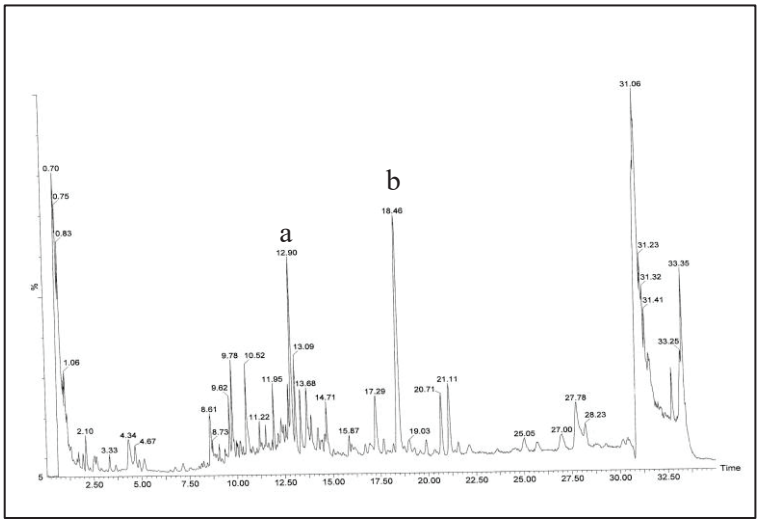
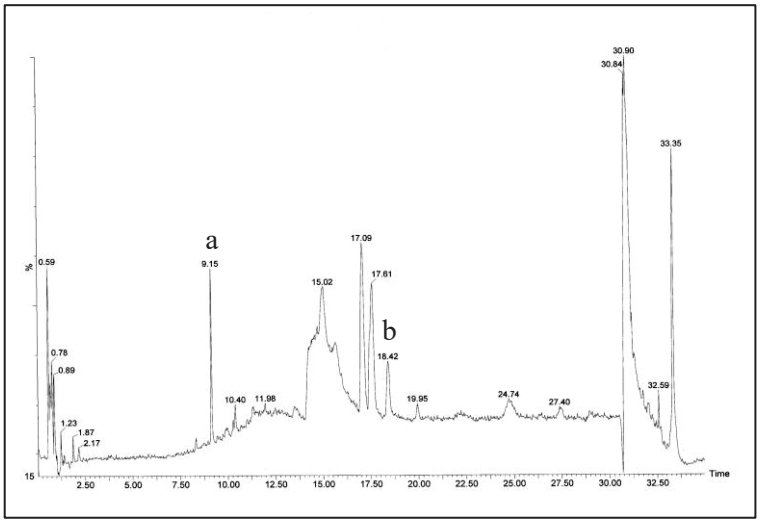


AMF

Fig. 44. Influence of AMF colonization on secondary metabolites in lemon balm water extract. Here, Cont, control plants; AMF, mycorrhizal plants; a, luteolin; b, kaemferol.



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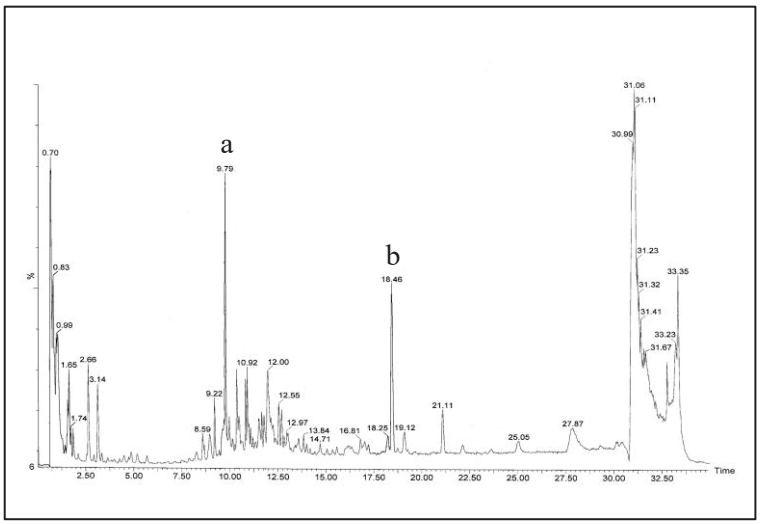
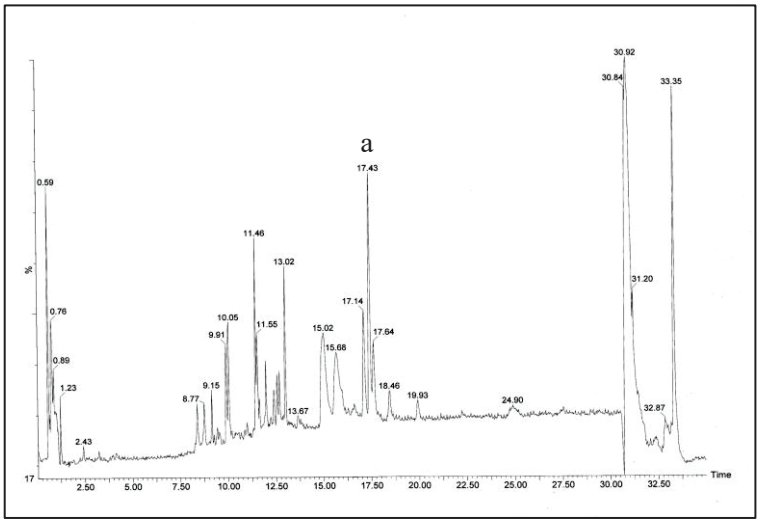


AMF

Fig. 45. Influence of AMF colonization on secondary metabolites in oregano water extract. Here, Cont, control plants; AMF, mycorrhizal plants; a, geniposide; b, kaemferol.



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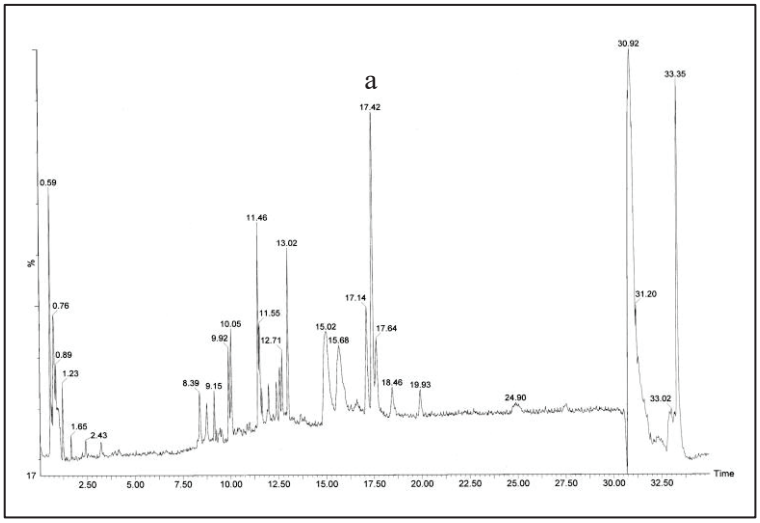
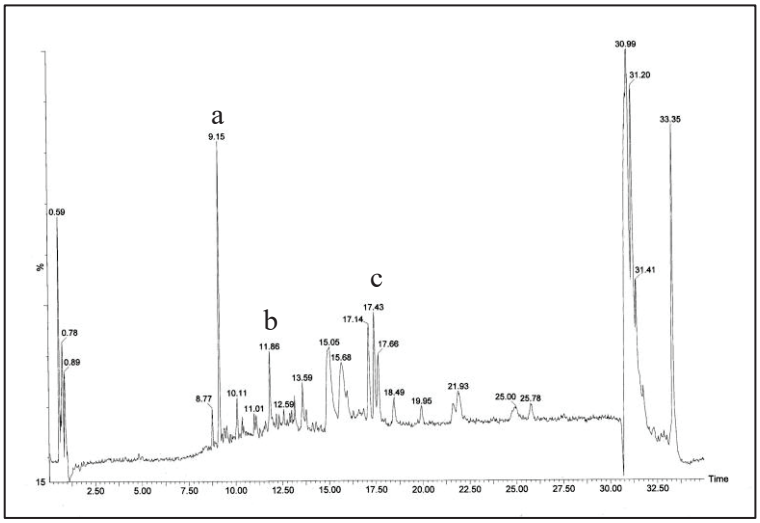


AMF

Fig. 46. Influence of AMF colonization on secondary metabolites in hyssop water extract. Here, Cont, control plants; AMF, mycorrhizal plants; a, kaemferol.



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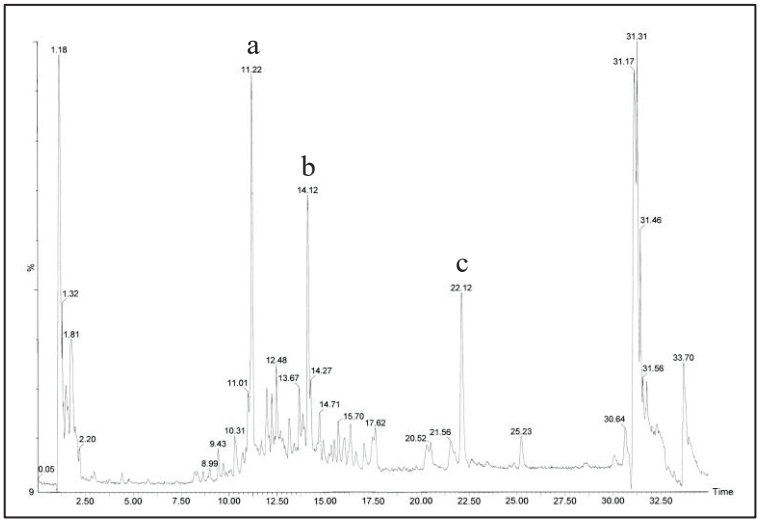
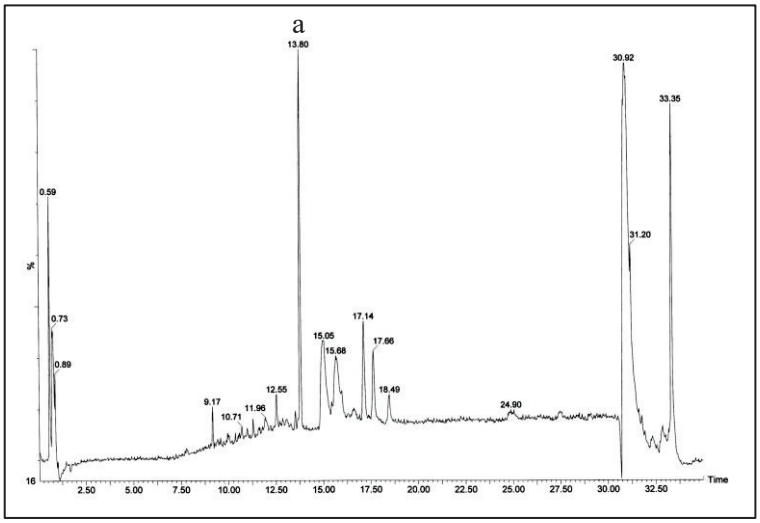


AMF

Fig. 47. Influence of AMF colonization on secondary metabolites in sage water extract. Here, Cont, control plants; AMF, mycorrhizal plants; a, geniposide; b, apigenin; c, kaemferol.



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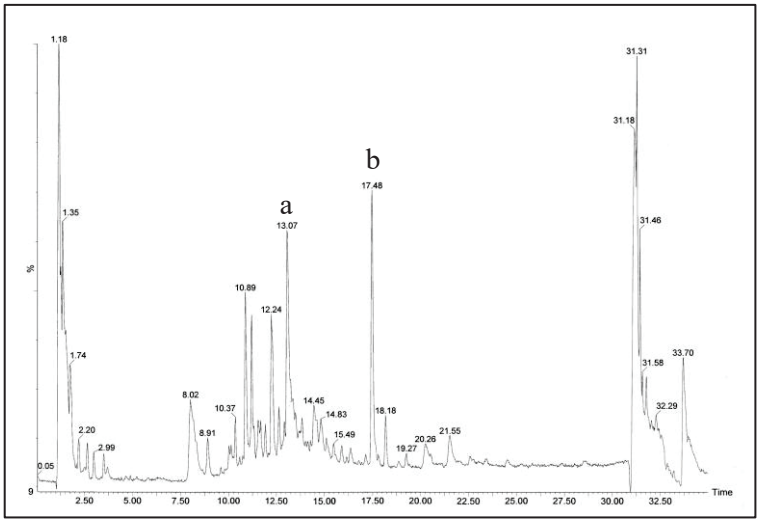
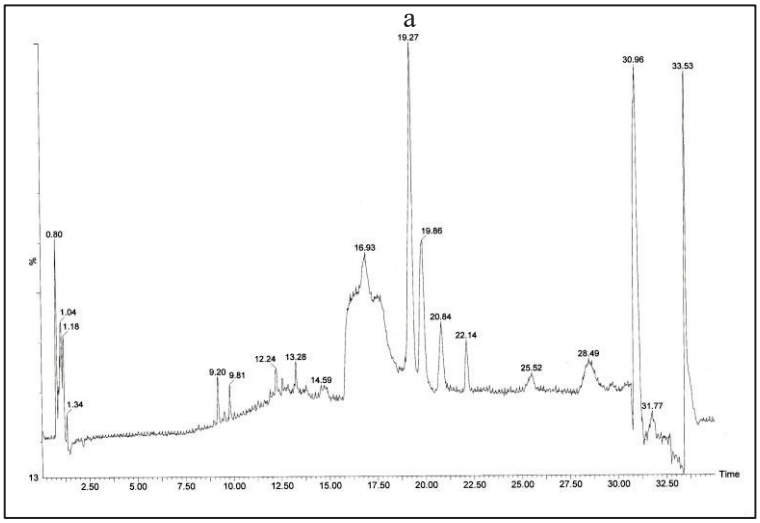


AMF

Fig. 48. Influence of AMF colonization on secondary metabolites in basil water extract. Here, Cont, control plants; AMF, mycorrhizal plants; a, change in the caffeic acid content; b. newly identified compound in AMF plot (coumric acid).



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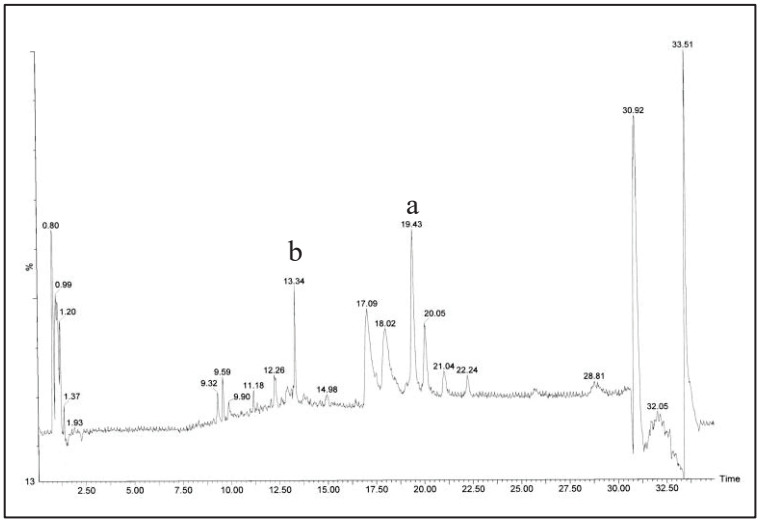

AMF

Fig. 49. Influence of AMF colonization on secondary metabolites in peppermint water extract. Here, Cont, control plants; AMF, mycorrhizal plants; a, change in the apigenin content; b, newly identified compound in AMF plot (Glucosinolate).

Discussion

The herbs under the family *Lamiaceae* have been widely used various industries due to containing many bioactive substances that have antioxidative, antimicrobial, anti-inflammatory, antifungal, antitumor and chemo preventive activities (Agostini et al., 2009; Rehan, 2014). More recently, the extracts of these herbs have found their use in controlling plant pathogenic organisms. As such, improving the quality of these herbs could potentially increase their practical use in various sectors. One of the ways to achieve this result is through application of AMF. AMF has the ability to improve the quality of host plant by improving the nutritional status and promoting the accumulation of effective ingredients (Zeng et al., 2013). From the results of the present study, it could be confirmed that treatment of herb plants with AMF boosted their growth as observed from the increased dry weights of shoots and roots. Enhancement of growth after inoculation by AMF has been reported on various plant species (Freitas et al., 2004; Copetta et al., 2006). The way this positive effect is achieved is primarily by the extension of the root penetration zone of the host plant which acts as an additional absorption surface (Sharma and Adholey, 2004). This increase results in the active uptake of more nutrients especially the immobile P, Zn and Cu (Phiri et al., 2003). Other ways of influencing growth might be through influencing hormone production, extracellular production of antibiotics and induction of plant systemic resistance to stress (Fillion et al., 1999). Improvement of herb growth promotion by AMF treatment was also reported by other researchers previously (Devi and Reddy, 2002; Copetta et al., 2006; Nell et al., 2009).

Regarding colonization level, the herbs under study did not show a considerable variation among them except for lemon balm. Several other researchers had also reported that the medicinal plants under *Lamiaceae* family are colonized with large variety of AMF (Copetta et al., 2006; Nell et al., 2009; Lee and Scagel 2009; Karagiannidis et al., 2010). The increased

colonization level of lemon balm could be result of its high affinity towards AMF as reported by Engel et al. (2016).

Application of AMF in the herb plants showed a considerable increase in the content of major secondary metabolites identified in the water extracts of each herbs. Furthermore, presence of new compounds in herbs with AMF treatment which was absent in control also implied the influence of such association on quality improvement of medicinal herbs. Studies had been undertaken before to evaluate the efficacy of the AMF on changes in secondary metabolites in plants particularly in roots (Larose et al., 2002; Zhu and Yao, 2004). On the other hand, influence of AMF on alteration of secondary metabolites in other part of plants had also been reported (Copetta et al., 2007; Ceccarelli et al., 2010; Kapoor et al., 2002). More recently, importance of AMF treatment on improving the functional components of medicinal plants have also come to light including herbs under *Lamiaceae* family (Zeng et al., 2013). However, most of the researches focused on the essential oils and the constituents of the essential oils like terpenes and terpenoids (Kapoor et al., 2002; Kapoor et al., 2004; Jurkiewicz et al., 2010). The findings of the present study indicated that AMF inoculation in *Lamiaceae* herbs like lemon balm, oregano, sage, hyssop, basil and peppermint could also modify the content of the water-soluble secondary metabolites. Contrary to the findings of Lee and Scagel (2009), we observed significant change in the polyphenol content of basil under AMF treatment with the identification of a new compound not present in non-AMF plants. Usually, the phenolic compounds present in these herbs are hydrophilic in nature and as such are found abundantly in aqueous extracts. Especially flavonoids and coumarins are most commonly found active ingredients in these medicinal plants. The AMF treatment seemed to have a positive effect in all the herbs under study; increasing the active component of the extracts as observed by higher peak size or generation of new compound. The increase in secondary metabolites content is considered to be the result of defense response of plants against fungal

colonization. However, the exact mechanism of such changes by AMF in plants is still unclear (Toussaint et al., 2007). Generally, the beneficial effect of AMF colonization is associated with increased P absorption by plants (Smith and Read 2008). Reports about increased secondary metabolites production by medicinal plants under P fertilization had been reported previously (Abu-Zeyad et al., 1999; Kapoor et al., 2004). Nell et al. (2009) observed a 1.2-fold increase in total phenolic and rosmarinic acid content in leaves of *S. officinalis* as compared to half phosphorus treatment, AMF inoculation or control plants. On the contrary, Toussaint et al. (2007) reported that increase of secondary metabolites like phenolic acids in basil could not be explained properly through P treatment. Some authors suggest that higher production of these secondary metabolites may be related to an increased activity of several enzymes like chalcone synthase and chalcone isomerase which are involved in synthesis of flavonoids and phenylalanine ammonia-lyase (PAL) (Lister et al., 1996; Ibrahim and Jaafar, 2011).These are responsible for the catalyzation of phenylalanine, which regulates the formation of phenolic compounds and biotic factors like colonization by AMF might increase the activity of such phenolic compound synthesis process. Beside this, AMF is reported to cause cytological change in host plants by increasing the number of plastid and mitochondria, resulting in the activation of tricarboxylic acid cycle and the plastid biosynthetic pathways, and ultimately increase the production of primary and secondary metabolites (Walter et al., 2000; Lohse et al., 2005; Strack and Fester 2006). Furthermore, the AMF stimulate the photosynthetic activity by the increase of photosynthetic pigment levels and by the drain of carbon resulting a higher export of triose phosphate to the cytoplasm with a larger activation of the Calvin cycle which results in a higher production of primary metabolites, which are precursors of secondary metabolism pathways (Kaschuk et al. 2009).

In conclusion, AMF treatment of the *Lamiaceae* herbs under the study showed a significant growth improvement in the plants as observed in the result. Furthermore, the UPLC-MS

analysis confirmed that such association had the potential to improve the quality of the water extracts of these herbs by improving/adding major secondary metabolites in it. This provides an eco-friendly way of improving the quality of *Lamiaceae* herbs and their water extracts which could further boost their use in disease control and growth promotion of horticultural crops. Beside this, the synergistic use of herb extract and AMF treatment on disease suppression of crops and possible induction of disease tolerance could be a possible avenue to pursue in future.

CHAPTER 3-2

Effect of lemon balm water extract on tolerance to anthracnose and antioxidative ability in mycorrhizal strawberry

Introduction

Colletotrichum gloeosporioides is a serious disease-inducing organism that affects the production cycle of strawberry in different growth stages, causing huge losses in major strawberry producing regions worldwide (Smith, 2008). Latent infection in the runners used, difficulty in developing resistant cultivars due to polyploidy, incomplete resistance of the developed cultivars, and inadequate control through cultural control methods make this disease a serious problem in strawberry cultivation. Generally, the use of synthetic fungicides is the primary control measure used against the disease at the producer level. However, these chemicals pose a major threat to the environment as well as to humans because of their low selectivity and lack of biodegradability (Gao et al., 2017). Hence, the search for an alternative and environment-friendly approach for disease control has become the present challenge in strawberry production.

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, essential oils (EOs) of lemon balm have applications in pharmacology, phytopathology, and food preservation (Abdellatif et al., 2014). The beneficial characteristics of lemon balm can be attributed to the phenolic compounds, terpenoids, and glucosides present in the extracts, which act as antioxidants (Weerakkody et al., 2011). However, the use of EOs under field conditions for disease control is impractical because of their innate volatile nature, which causes them to spontaneously diffuse, which results in a decrease in the active concentration for disease suppression (Letessier et al., 2001). On the other hand, the water extract of lemon balm had a similar disease control effect due to the presence of antifungal secondary metabolites, especially rosmarinic acid, and a longer active period due to its nonvolatile nature (Ahmad and Matsubara, 2020a). Therefore, an eco-friendly disease control measure could be envisioned from this.

In response to the constant challenge from different biotic and abiotic stresses, plants have developed a wide range of strategies to protect themselves. A prime example of such is the association between plants and arbuscular mycorrhizal fungi (AMF), which can be found in the roots of almost 80% terrestrial plant species (Baum et al., 2015). Besides supplying nutrition, this association causes remarkable changes in the physiology of the host plant (Schliemann et al., 2008; López-Ráez et al., 2010). Such changes have a considerable impact on a plant's responses to different abiotic and biotic stresses (Smith et al., 2010; Campos-Soriano et al., 2012). These findings highlight the importance of the AMF associations in the search for a sustainable and eco-friendly alternative to conventional disease control measures.

A high concentration of reactive oxygen species (ROS), such as H_2O_2, superoxide anion (O_2^-) and hydroxyl radical (OH^-), is produced in plants as a defense mechanism upon pathogen invasion, which is a phenomenon known as an oxidative burst. To overcome the negative impact of ROS, plants have evolved protective measures that eliminate these ROS partially or completely through the production of antioxidants (Moghaddam et al., 2006). However, the excessive production of pathogenesis-related ROS can overwhelm these antioxidant defenses, leading to severe damage to plants (Sahoo et al., 2007). Therefore, enhancing the antioxidative ability of plants can improve their disease resistance by scavenging excess ROS as a part of induced systemic resistance. The promotion of antioxidative functions, such as higher superoxide dismutase activity and increased ascorbic acid content, were reported in mycorrhizal plants (Richter et al., 2011). Previously, it was reported that mycorrhizal strawberry plants expressed higher tolerance to anthracnose than non-mycorrhizal plants, and this phenomenon was attributed to the higher antioxidative ability of the mycorrhizal plants (Li et al., 2010). In contrast, phytoextracts contain a complex mixture of different compounds or metabolites and, when used as a treatment, can influence a plant's development and response to stress, both directly and indirectly (Miraj et al., 2017). Therefore, along with a direct

antifungal effect against anthracnose, lemon balm extract may also influence disease tolerance in plants through secondary pathways, such as antioxidative defense. However, information regarding the potential use of water extracts of lemon balm for this is scarce. Furthermore, the use of both AMF associations and lemon balm water extract might provide a synergistic disease tolerance effect, which is a potential option to consider. Therefore, the following experiment was conducted to determine the impact of lemon balm water extracts on the tolerance to anthracnose and antioxidative ability in mycorrhizal strawberry plants.

Materials and Methods

Growing lemon balm and preparation of the water extract: Lemon balm (*Melissa officinalis* L.) seeds 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 were 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 the herb extract solution.

Plant material and arbuscular mycorrhizal fungi inoculation: Two-month-old strawberry (*Fragaria* × *ananassa* Duch., cv. Tochiotome, susceptible to anthracnose) runner plants were planted in pots (10.5 cm in diameter, 0.5 L) containing autoclaved commercial soil (SM-2, Premier Tech., Canada) and fertilized using a slow-release granular fertilizer (long total 70-day type; N:P:K = 13:9:11, JCAM Agri. Co. Ltd., Japan) (Fig. 50). At the time of transplanting, the plants were inoculated with 5 g of commercial AMF inocula (*Gigaspora margarita*, GM; *Glomus fasciculatum,* Gf; obtained from Centralgrass Co. Ltd. and Idemitsuagri Co. Ltd. Japan, respectively; spore densities unknown), according to Li et al. (2010). The non-AMF plants received the same amount of autoclaved (121°C, 1.2 kg/cm^2, 30 min) inocula. Ten plants per

treatment in triplicates were grown in a greenhouse at 30/24 ± 4°C day/night temperatures, with a 12–13 h photoperiod (750–1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and 70%–80% relative humidity (natural conditions).

Application of lemon balm water extract and pathogen inoculation: Ten weeks after AMF inoculation, the water extract (20%, w/v) of lemon balm was sprayed (Lb; 10 ml/plant) on strawberry plants twice (on subsequent days) before pathogen inoculation (Fig. 50). For the control plants, distilled water was used. *Colletotrichum gloeosporioides* (*C. fructicola*, CG1) were cultivated on potato dextrose agar medium and incubated in dark conditions at 25°C for two weeks to facilitate sporulation; they were further subcultured for 7–10 days to facilitate more sporulation. The spores were harvested in distilled water, and the concentration was adjusted to 10^5 conidia/ml. Each strawberry plant was sprayed with 10 ml of the conidial suspension immediately after the second application of the herbal extracts and was then covered with plastic film for the first week to maintain humid conditions around the plant to facilitate inoculation (at 30/24 ± 4°C day/night temperature). Two weeks after the inoculation, the symptoms of anthracnose were assessed as described by Li et al. (2010); that is, the percentage of diseased leaves and petioles were classified using 5 levels: Level 1, < 20%; Level 2, 20%–40%; Level 3, 40%–60%; Level 4, 60%–80%; Level 5, 80%–100%. The disease index was calculated using the following formula:

$$\text{Disease index} = \frac{\sum (\text{number of plants} \times \text{number of degree in symptoms})}{\text{Total number of plants} \times \text{maximum degree in symptoms}} \times 100$$

Evaluation of arbuscular mycorrhizal fungi colonization and plant growth: Twelve weeks after AMF inoculation (including two weeks of CG1 inoculation), all plants were uprooted. The lateral roots of the uprooted strawberry plants were preserved with 70% ethanol and stained according to the method of Phillips and Hayman (1970). The percentage of AMF colonization in 1 cm segments of the lateral roots was calculated using approximately 50

samples of 1 cm segments per plant from 10 randomly selected plants. For the dry weight analysis, ten plants were randomly chosen from each treatment and separated into shoots (compatible leaves and petioles), crown, and roots. The roots were cleaned carefully under slow-flowing tap water in a tray to remove soil and debris while preventing the loss of fine roots. The roots were then dried using a constant temperature drier (ETTAS 600B) at 80°C for two days. The dry weights of the shoots and roots were then measured. The remaining plants were cryopreserved for antioxidative analysis.

Determination of antioxidative abilities:

Measurement of superoxide dismutase (SOD) activity: Measurement of SOD activity was carried out according to the method of Beauchamp and Fridovich (1971) (Fig. 51). Frozen sample (0.1 g) was extracted using 3 ml of 50 mM phosphate buffer (pH 7.0) and the extract was centrifuged at 13,000 rpm, 4°C, 10 min. Then the supernatant (0.1 ml) was mixed with 2.3 ml of 50 mM sodium carbonate buffer (pH 10.2), 0.1 ml of 1.0 mM NBT (nitro blue tetrazolium), 0.1 ml of 4.0 mM xanthine, 0.1 ml of 3.0 mM EDTA (ethylenediaminetetraacetic acid), 0.1 ml of 0.15% (w/v) BSA (bovine serum albumin), 0.1 ml of 12.5 units/ml xanthine oxidase (100-fold diluted solution). The mixture was then allowed to stand at room temperature for 30 minutes in dark condition. Thereafter, 0.2 ml of 14 mM $CuCl_2$ [copper (II) chloride] solution was added and the absorbance of the reaction mixture was measured at a wavelength of 560 nm with a spectrophotometer. The one unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25ºC.

Measurement of ascorbate peroxidase (APX) activity: The ascorbate peroxidase (APX) activity was measured according to Nakano and Asada (1981), where 3 ml of the reaction mixture had 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM H_2O_2, and 0.1 mM enzyme added (Fig. 52). Then, 0.1 mM H_2O_2 was added to

initiate the reaction, and a decrease in absorbance was measured at 290 nm using the spectrophotometer for 1 min.

Measurement of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability: The DPPH radical scavenging ability was measured according to the method of Burtis and Bucar (2000) (Fig. 2). Frozen sample (0.1 g) was extracted using 3 ml of 80% ethanol and the extract was centrifuged at 13000 rpm, 4°C, 10 min. Then the supernatant (0.15 ml) was mixed with 0.9 ml of 400 μM DPPH solution, 0.9 ml of 0.2 M MES buffer solution (pH 6.0), 0.9 ml of 20% ethanol and 0.75 ml of 80% ethanol. Then the mixture was allowed to stand at room temperature for 30 min in dark condition. At that time, a blank was prepared by adding 0.15 ml of 80% ethanol. After completion of the reaction, the absorbance at 520 nm was measured with a spectrophotometer. Trolox (10 to 100 μg/ml) was used to create a calibration curve, and the blank measurement value was subtracted from the measurement value of the analysis sample, and the value calculated on the calibration curve was taken as the DPPH radical scavenging ability.

Polyphenol content determination: The method of MacDonald et al. (2001) using Folin-Denis reagent was adopted for the determination of the polyphenol contents in the prepared extracts, and absorbance was measured at 700 nm (Fig. 3). Frozen sample (0.1 g) was extracted by using 4 ml of 80% methanol and centrifuged at 13,000 rpm, 4°C, 10 min. Then the supernatant (0.2 ml) was mixed with 2.4 ml of distilled water, 0.2 ml of a Follin Denis reagent [distilled water 70 ml, sodium tungstate dihydrate 10 g, phosphomolybdic acid [12 molar (IV) phosphoric acid n-hydrate 2 g], 5 ml phosphoric acid and 0.4 ml saturated sodium carbonate. Then the mixture was allowed to stand in dark condition at 30ºC for 30 minutes. At that time, a blank was prepared by adding 0.2 ml of distilled water instead of Folin-Denis reagent. After completion of the reaction, the absorbance of the reaction mixture was measured at a wavelength of 700 nm using a spectrophotometer. Quercetin (1 to100 μg/ml) was used for the

calibration curve preparation, and the blank value was subtracted from the measurement value of the sample solution, and the value calculated from the calibration curve was taken as the polyphenol content.

Ascorbic acid (AsA) content: For ascorbic acid, samples were extracted using 5% metaphosphoric acid at a ratio of 0.15 g/5 ml and analyzed as described by Mukherjee and Choudhuri (1983) (Fig. 4). Then the supernatant (0.5 ml) was mixed with 0.5 ml of 0.03% DCIP (sodium 2,6-dichloroindophenol solution), 0.5 ml of a 2% thiourea-5% metaphosphoric acid solution and 0.25 ml of 2% DNP (2,4-dinitrophenylhydrazine) solution. Then the mixture was kept in water bath at 50ºC for 70 min. After completion of the reaction, 2.0 ml of 85% sulfuric acid was slowly added while cooling in ice and the mixture was allowed to stand in dark condition at room temperature for 30 minutes. At the same time, the test tube which was used as blank (2% DNP solution) was also kept in dark condition but without adding the sulfuric acid solution. Then the absorbance of the reaction mixture was measured at a wavelength of 520 nm using a spectrophotometer. For the calibration curve creation, L-ascorbic acid (1 to100 µg/ml) was used, and the value obtained by subtracting the blank value from the measurement value of the sample solution was applied to the calibration curve to calculate the ascorbic acid content.

Statistical analyses: The mean values for the dry weights of shoots and roots, colonization percentage, disease index, DPPH radical scavenging activity, SOD activity, APX activity, AsA content, and polyphenol content were analyzed by Tukey's multiple range test, which significance determined at $P < 0.05$. All analyses were conducted using XLSTAT 2012 pro statistical analysis software (Addinsoft, New York).

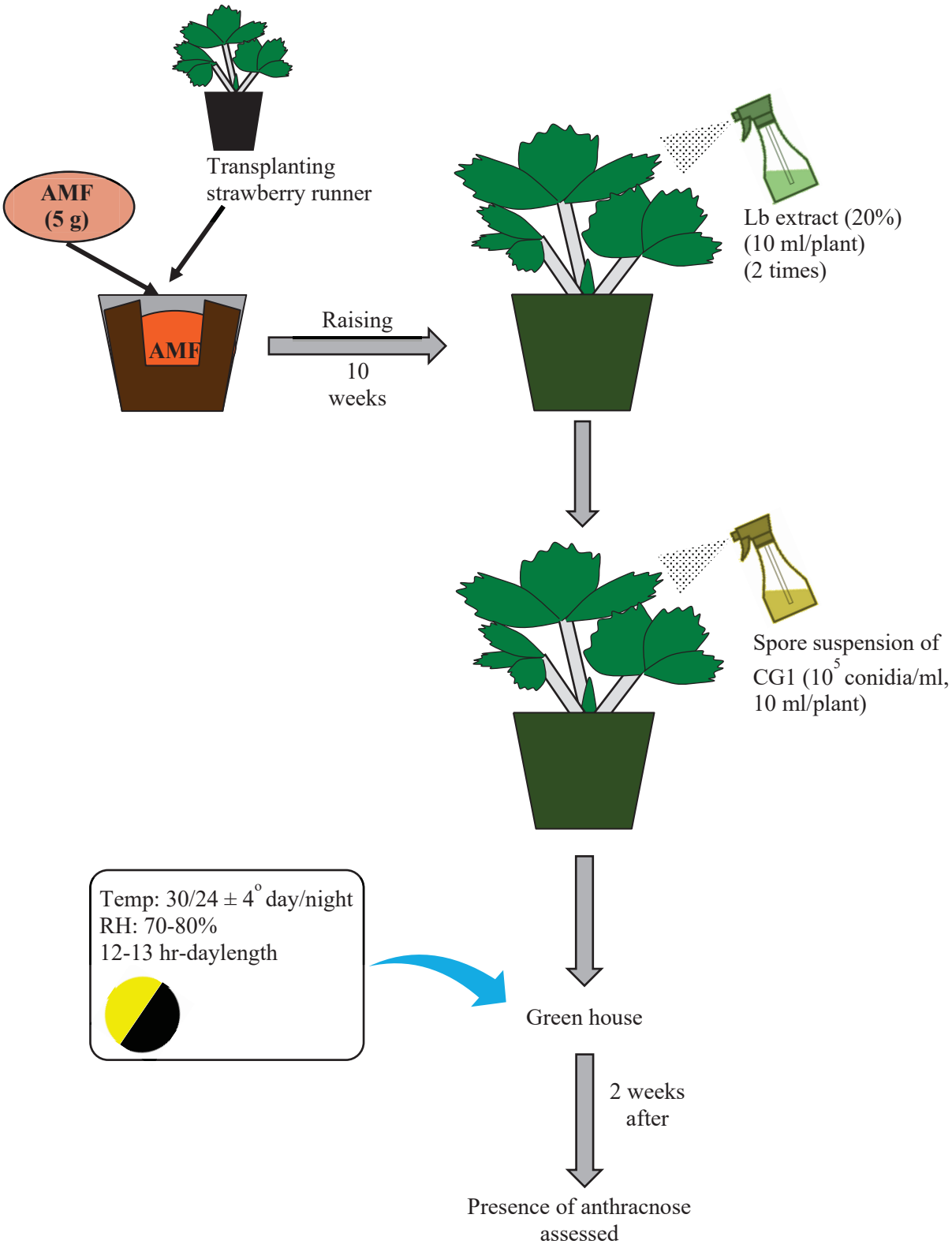

Fig. 50. AMF application, herb extract treatment and CG1 inoculation in strawberry plants. Here, AMF, arbuscular mycorrhizal fungi; Lb, lemon balm; CG1, *Colletotrichum gloeosporioides* strain CG1.

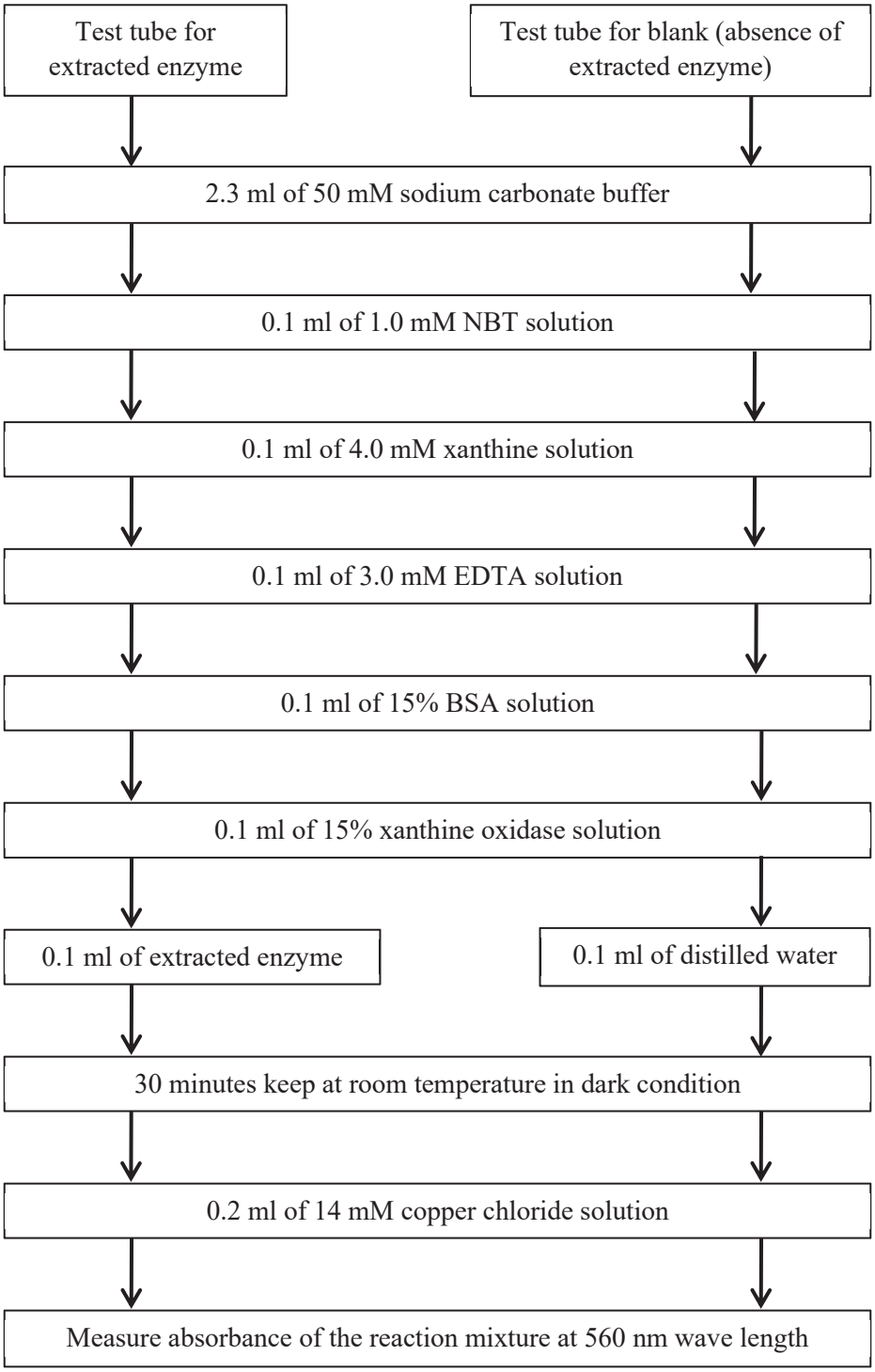

Fig. 51. Flow diagram of the procedures in SOD analysis.

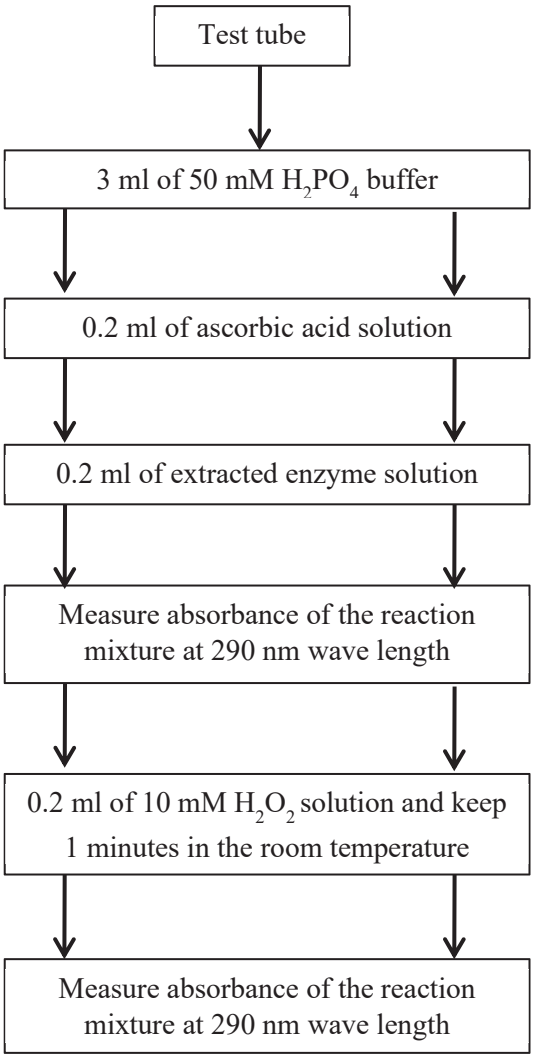

Fig. 52. Flow diagram of the procedures in APX analysis.

Results

Two weeks after the CG1 inoculation, the incidence of anthracnose in the control plants reached 100% and was highest among the treatments, with a severity level of 4 (Fig. 53A). In contrast, plants under the AMF treatment showed a lower incidence rate, with none reaching 100%. Among the two AMF species, the lowest severity level was observed in plants under the GM treatment (Level 2). No significant difference was observed in the disease indices between the two AMF species (Fig. 53B). Plants treated with only Le also showed a lower disease incidence compared to the control and were not significantly different compared with the AMF species regarding disease indices. In the combination treatments, the disease indices decreased significantly compared to the other treatments, especially under the GM + Le treatment (11.4), which had a lower severity level (level 1).

Twelve weeks after the AMF treatment (including two weeks after the CG1 inoculation), the strawberry plants under GM + Le treatment had significantly higher shoot dry weights compared to the control (Fig. 54). Both the Gf + Le and GM + Le treated plants expressed significantly higher root dry weights compared to the control. The root and shoot dry weights of the other treatments were not significantly different compared to the control.

Regarding the AMF colonization level, no significant difference was observed between the two species used, regardless of the use of the Le treatment (Fig. 55).

Both the AMF colonization and Le treatment had a considerable effect on the antioxidant activity of the strawberry plants. Concerning the enzymatic antioxidants such as SOD and APX, there was increased activity compared to the control when Le and AMF treatments were applied separately (Fig. 56). The GM treatment resulted in higher SOD and APX activity in the shoots compared to Gf, although no significant difference in activity was present in the roots. Plants treated with Le showed statistical similarity with Gf in shoot SOD activity and

with GM in shoot APX activity. In the case of the combination treatment, significant increases in SOD and APX activity were observed in plants under the GM + Le treatment in both the shoots (226.7 unit/g fresh weight (FW) and 0.7 units/g FW for SOD and APX, respectively) and roots (333.3 unit/g FW and 0.6 units/g FW for SOD and APX, respectively). However, the GM and Gf + Le treatments were statistically similar to GM + Le in the shoot SOD activity (218.2 unit/g FW and 215.6 unit/g FW for GM and Gf + Le, respectively).

Regarding non-enzymatic antioxidative properties, increased DPPH radical scavenging activity and higher AsA and polyphenol content were observed in the AMF and Le treatments in both shoots and roots compared to the control (Fig. 57). However, no significant difference in DPPH scavenging activity was observed between the two AMF species or between these and the Le treatment. On the other hand, the AsA and polyphenol contents were higher in plants under the GM treatment than in Gf. Plants under the combination treatment showed the highest DPPH radical scavenging activity among all the treatments, with the maximum observed in the shoots of plants under the GM + Le treatment (61.1 mg/g FW) and in the roots of those under the Gf + Le treatment (58.0 mg/g FW). In both cases, the two combination treatments were statistically similar each other. Regarding the AsA and polyphenol content, the highest concentrations were observed in strawberry plants under the GM + Le treatment for both shoots (66.8 mg/g FW and 173.6 mg/g FW for AsA and polyphenol, respectively) and roots (13.8 mg/g FW and 160.9 mg/g FW for AsA and polyphenol, respectively).

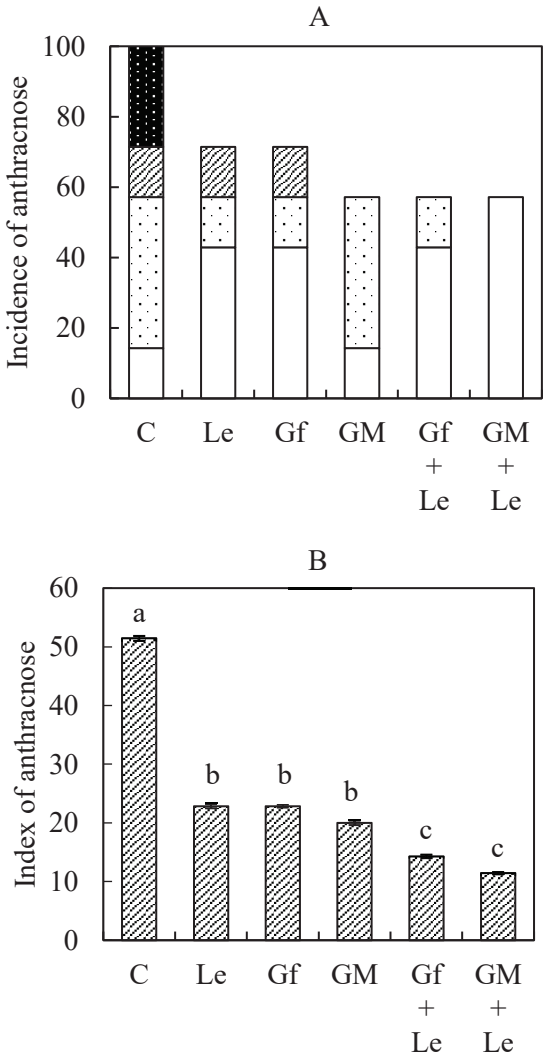

Fig. 53. Inhibitory effect of AMF colonization and lemon balm water extract on disease incidence (A) and indices (B) in strawberry two weeks after CG1 inoculation. Co, control; Le, lemon balm; Gf, *Glomus fasciculatum*; GM, *Gigaspora margarita*; Gf+Le, inoculation by *Glomus fasciculatum* and treated with lemon balm extract; GM+Le, inoculation by *Gigaspora margarita* and treated with lemon balm extract. Here, □, <20; ⬚, 20–40; ▨, 40–60; ▪, 60–80; ■, 80–100%. Columns denoted by different letters indicate significant difference according to Tukey's test ($P < 0.05$).

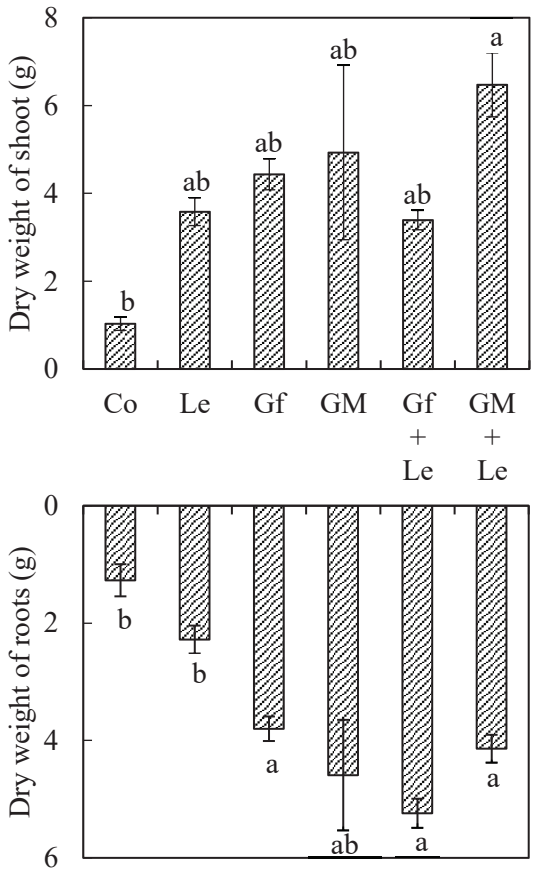

Fig. 54. Dry weight of shoots and roots in strawberry plants 2 weeks after CG1 inoculation. Co, control; Le, lemon balm; Gf, Glomus fasciculatum; GM, Gigaspora margarita; Gf+Le, inoculation by Glomus fasciculatum and treated with lemon balm extract; GM+Le, inoculation by Gigaspora margarita and treated with lemon balm extract. Columns denoted by different letters indicate significant difference according to Tukey's test ($P < 0.05$).

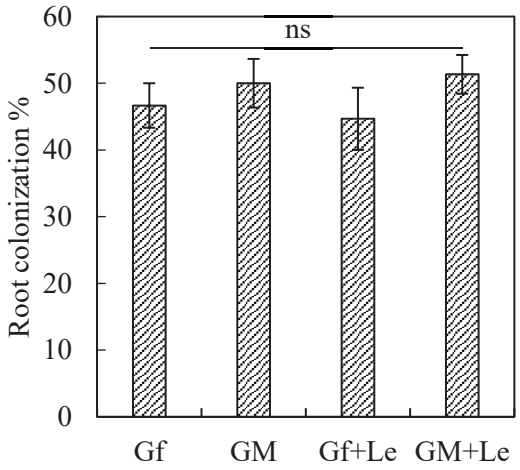

Fig. 55. Percentage of root colonization by the AMF species twelve weeks after inoculation. Gf, *Glomus fasciculatum*; GM, *Gigaspora margarita*; Gf+Le, inoculation by *Glomus fasciculatum* and treated with lemon balm extract; GM+Le, inoculation by *Gigaspora margarita* and treated with lemon balm extract. ns, no significant difference according to Tukey's test ($P < 0.05$).

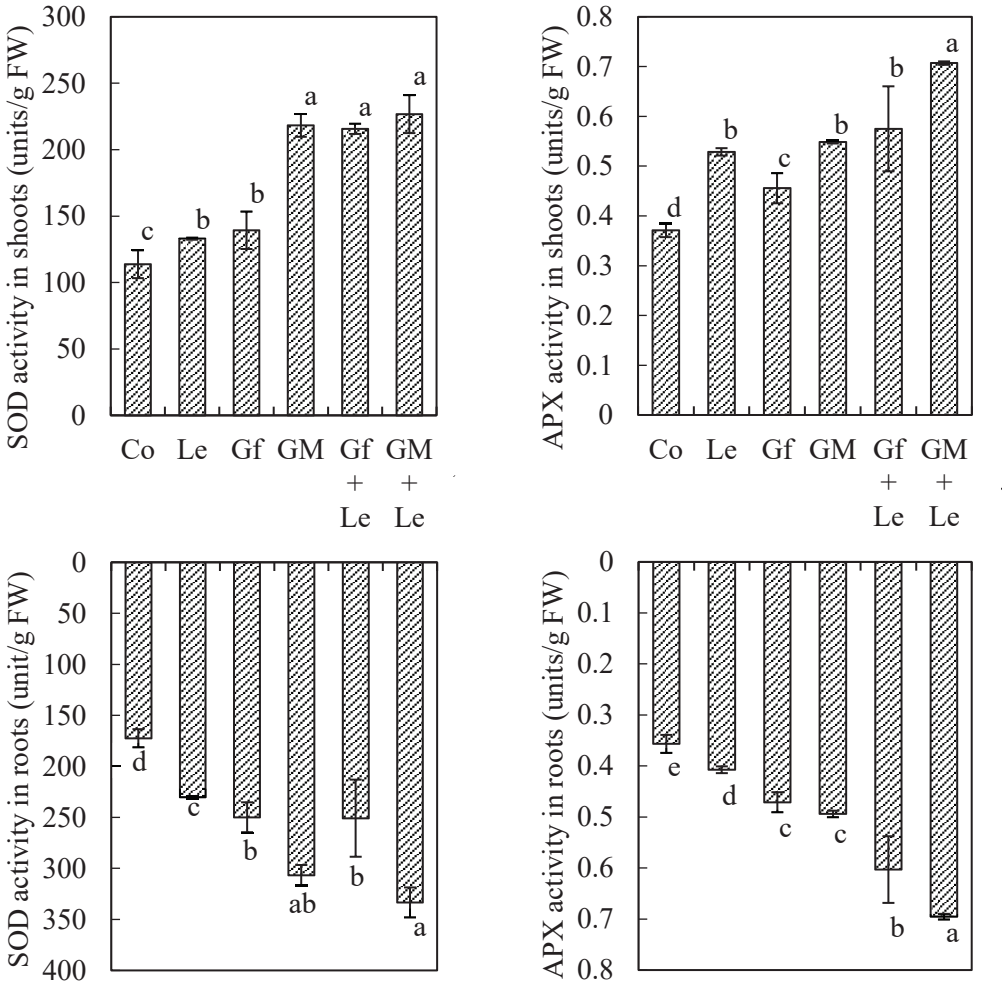

Fig. 56. Enzymatic antioxidant activity (SOD and APX) in shoots and roots of mycorrhizal strawberry plants under lemon balm treatment 2 weeks after CG1 inoculation. Co, control; Le, lemon balm; Gf, *Glomus fasciculatum*; GM, *Gigaspora margarita*; Gf+Le, inoculation by *Glomus fasciculatum* and treated with lemon balm extract; GM+Le, inoculation by *Gigaspora margarita* and treated with lemon balm extract. Columns denoted by different letters indicate significant difference according to Tukey's test ($P < 0.05$).

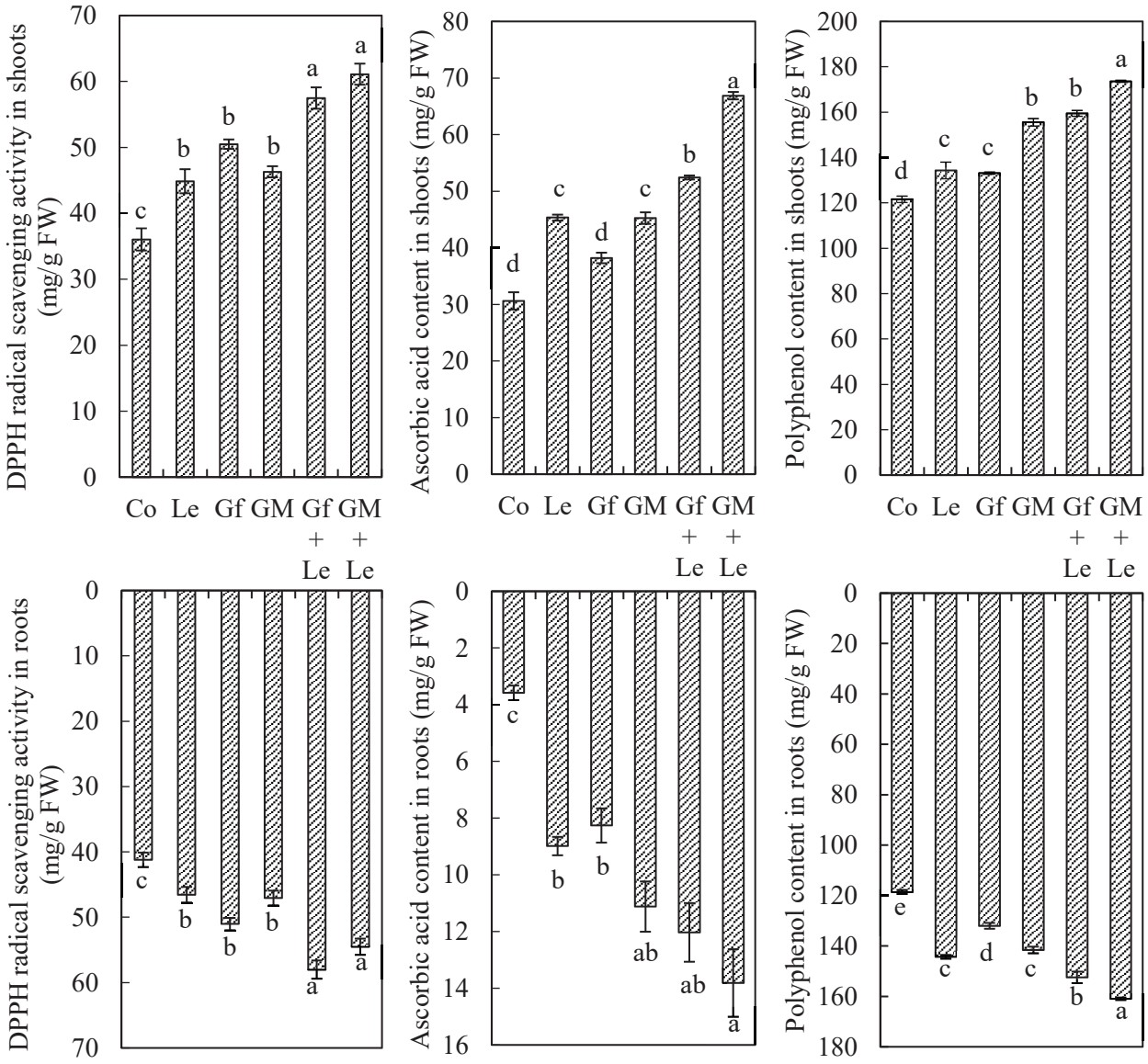

Fig. 57. Non-enzymatic antioxidant content (DPPH radical scavenging activity, AsA and polyphenol) in shoots and roots of mycorrhizal strawberry plants under lemon balm treatment 2 weeks after CG1 inoculation. Co, control; Le, lemon balm; Gf, *Glomus fasciculatum*; GM, *Gigaspora margarita*; Gf+Le, inoculation by *Glomus fasciculatum* and treated with lemon balm extract; GM+Le, inoculation by *Gigaspora margarita* and treated with lemon balm extract. Columns denoted by different letters indicate significant difference according to Tukey's test ($P < 0.05$).

Discussion

In the wake of the severe detrimental effects of synthetic agrochemicals on the environment and human health, the search for eco-friendly and economically viable solutions against abiotic and biotic stresses in crop production has become a prime need. In the present study, the incidence of anthracnose in strawberry plants treated with AMF was considerably lower than that in the control plants, which consequently expressed lower disease indices. According to Li et al. (2010), strawberry plants under the AMF treatment were shown to have a relatively higher tolerance to anthracnose than non-AMF plants. Our results also indicated a similar tolerance when compared with the control. Arbuscular mycorrhizal fungi have been reported to suppress pests and diseases through induced systemic resistance (ISR), which is not related to the improved nutritional status of the host plant (Fritz et al., 2006). The colonization effect from AMF acts as a stimulator of the defense mechanisms of the host plant, a process called priming. This priming results in plants to be in an alert condition, such that the defense response of the primed plant against pathogen invasion occurs more swiftly compared to non-primed plants (Jung et al., 2012).

Plants treated with the lemon balm extract also showed a reduced disease incidence and index compared to the control. This could be the result of a direct antifungal effect, as shown in our previous study (Ahmad and Matsubara, 2020b) owing to the presence of antifungal secondary metabolites, such as rosmarinic acid and luteolin, in the water extract. However, extracts from a plant source such as lemon balm are usually a complex mixture of phytochemicals or metabolites that can have a multitude of effects (Miraj et al., 2017). Therefore, a secondary pathway of disease suppression might be present, in addition to the direct antifungal effect of the lemon balm extract in strawberries.

During a pathogen invasion, plants start to produce an excessive amount of ROS to fight against the pathogen (oxidative burst), which can cause oxidative damage to the membrane, lipids, proteins, and nucleic acids in the plant cells (Srivalli et al., 2003). For this reason, a stimulatory effect is necessary for plants to boost their innate antioxidant activity to tackle these pathogenesis-related ROS. From the results of the present experiment, it was evident that AMF plants expressed a considerable increase in enzymatic antioxidants such as SOD and APX compared to the control. Li et al. (2010) also reported a similar increase in SOD and APX activity in mycorrhizal strawberry plants. Arbuscular mycorrhizal fungi promote antioxidant activity by utilizing various mechanisms such as (a) enhancing nutrient uptake, (b) increasing the efficiency of the host plants by increasing their growth, and (c) producing phytochemicals such as flavonoids (Mollavali et al., 2015). Furthermore, some reports emphasize that colonization increases the enzymatic antioxidant activity in plants (Wang et al., 2002). An increase in the SOD and APX activity in plants by AMF was also reported by Maya and Matsubara (2013) in cyclamen.

Regarding DPPH radical scavenging activity as a measure for non-enzymatic antioxidants, this, as well as the AsA and polyphenol contents, were increased in AMF plants compared to the control. The DPPH radical scavenging activity is a measure of non-enzymatic antioxidant activity and indicates a plant's ability to scavenge free radicals (Kang and Saltveit, 2002). Ascorbic acid is considered a powerful antioxidant due to its action as an electron donor in a wide range of enzymatic and non-enzymatic reactions (Das and Roychoudhury, 2014). On the other hand, polyphenols have lower electron reduction potential compared to oxygen radicals, resulting in direct scavenging of oxygen intermediates without an oxidative reaction (Ainsworth and Gillespie, 2007). Therefore, an increase in the DPPH radical scavenging activity, AsA contents, and polyphenol contents through the AMF association would give the plants a higher chance to prevent oxidative damage caused by pathogenesis-related ROS. In

this way, AMF could improve the plant's innate enzymatic and non-enzymatic antioxidant ability as a part of ISR, which translates into higher disease tolerance.

In our earlier experiment (Ahmad and Matsubara, 2020), the water extract of Lemon Balm was reported to contain rosmarinic acid and luteolin (confirmed by an ultra-performance liquid chromatography-tandem mass spectrometry analysis), which expressed a direct antifungal effect against CG1. However, they were also reported to act as strong antioxidants along with other hydroxycinnamic acids and flavonoids in aqueous extracts (Martins et al., 2012). Lemon balm infusion was reported to improve the plasma levels of catalase, SOD, and glutathione peroxidase and a marked reduction in plasma DNA damage, myeloperoxidase, and lipid peroxidation (Miraj et al., 2017). They were also found to suppress the formation of DPPH, hydroxyl, and lipid peroxyl radicals in a dose-dependent manner (Canadanović-Brunet et al., 2008). The findings of the present study also showed a marked increase in the antioxidative ability of strawberry plants under the lemon balm treatment. Therefore, the application of lemon balm extract, besides acting as a direct antifungal agent, could also elevate the antioxidative defense potential in strawberry plants, which leads to increased disease tolerance.

From the above discussion, it is apparent that the AMF and lemon balm extract treatment could be employed as an eco-friendly defensive measure against anthracnose of strawberry plants. As such, their combined utilization could potentially provide a more pronounced disease tolerance in strawberries, which was observed in the results. This is the first report of implementing such disease control measures in strawberry plants. Both enzymatic and non-enzymatic antioxidant properties were considerably enhanced in plants treated with the lemon balm extract under AMF. An increase in the antioxidative ability possibly helped the plants to scavenge more pathogenesis-related ROS than those under the control treatment, preventing oxidative damage to the cells and maintaining homeostasis. As such, the combination treatment expressed a stronger disease tolerance, as observed from the disease index results. The AMF

and herb extract treatment possibly complemented each other in the stimulation of the biochemical activities of the strawberry plants, causing higher DPPH radical scavenging activity, AsA contents, and polyphenol contents with greater SOD and APX activity. Furthermore, earlier reports regarding AMF colonization stated that even though colonization boosted host plant growth, it sometimes made the host plant susceptible to disease (Whipps, 2004). If we consider such a scenario, then using the lemon balm extract in conjunction with AMF could feasibly ameliorate this drawback, where AMF would boost plant growth, and the lemon balm extract would directly suppress the pathogen and stimulate disease tolerance.

Regarding the AMF colonization level, there was no clear evidence about the impact of this on disease tolerance. Ozgonen and Erkilic (2007) reported that in pepper, growth promotion and tolerance to *Phytophthora capsica* were not related to the level of AMF colonization. In our present experiment, no significant difference was observed in the colonization level between the AMF species, and no significant impact on disease tolerance could be determined based on their level of colonization. Furthermore, the use of the herb extract did not seem to interfere with AMF colonization. According to Widmer and Laurent (2006), *Lamiaceae* herb extracts such as rosemary, lavender, and sage were found to have no inhibitory effect on conidial germination of plant symbiotic fungi, making their use suitable for the synergistic control of diseases.

The findings of the present study suggest that treatment with the water extract of lemon balm in conjunction with AMF can increase the tolerance of strawberry plants to anthracnose disease by boosting innate defense mechanisms. The reduction in disease incidence and indices, along with increased antioxidative ability, highlight this. However, the specific mechanism of disease tolerance is yet to be clarified. Further experimentation should focus on elucidating the underlying mechanisms related to resistance induction in the AMF and herb extract-treated strawberry plants.

Chapter 3- Conclusion

From the findings of the experiments compiled in this chapter, it can be concluded that, the use of arbuscular mycorrhizal fungi could improve the quality of the herb plants through boosting their growth and increasing the important secondary metabolites present in them. Besides increasing the content of the major secondary metabolites, AMF treatment could also stimulate the generation of additional compounds in herbs that were not detected in control plants. The addition of such compounds might further improve the activity if the herb extracts when used on host plant. Furthermore, the new compounds might improve the use of herb extracts in a broad aspect. The synergistic use of both AMF and herb extract on suppression of anthracnose in strawberry suggested a more prominent disease control compared to single use of these treatments. Moreover, they showed induction of disease tolerance in the host plant as evidenced by the improved antioxidative potential. The use of such synergistic control measured provides us with a new way of controlling diseases in crops. Beside directly suppressing the disease, they also pave a way to improve the innate disease resistance quality of host plants, giving a more holistic approach in phytopathogen control.

CHAPTER 4

Changes in secondary metabolites and free amino acid content in tomato with *Lamiaceae* herbs companion planting

Introduction

Companion planting is an intercropping practice where two or more plants are grown together in a unit area. This practice of crop production is usually associated with organic agriculture especially in small scale farms or gardens which do not utilize much investment or technologies. The popularity of the companion planting method in garden production system is the result of popular books (Rittoe, 1975; Cunningham, 1998), newspaper articles and internet websites. The target of companion planting is that, the plants involved will interact with each other agronomically and also influence the microclimate surrounding them (Gómez-Rodríguez et al., 2003; Bomford, 2009). Besides influencing the microclimate, it is also used as a way of eco-friendly pest control method. That is, plants which directly mask the specific chemical cues are grown together to confuse the pests, or because they hold and retain particularly effective natural enemies of one another's pests (Parker et al., 2013). However, certain disadvantages could arise between the companions due to competition for light, water and nutrients which may cause yield reduction of the main crop (Lu et al., 2000; Jedrszczyk and Poniedzialek, 2007; Borowy, 2012). Still, garden producers have stood by their opinion that companion planting has the potential to be advantageous and to be adopted in conventional production. Adjustment of irrigation and fertilizer management (Kołota and Adamczewska-Sowi´nska, 2013), sowing companion crop with or after planting the main crop (Adamczewska-Sowi´nska and Kołota, 2008), selection of appropriate companion plant could possibly minimize the impact of competition in the companion planting system. Reports of increased yield in main crop under intercropped system through proper management is also present (Lu et al., 2000; Mandal and Dash, 2012).

One of the popular companion combinations mentioned by the gardeners is the tomato-basil companion planting (Riotte, 1975). It had been reported that basil acted as repellent of flies and improve the yield and taste of tomato when grown as companion (Bradley and Ellis,

1992). Positive influence on control of insect pest like mites, mosquitos and tomato hornworm was also claimed by producers in tomato-basil companion system (Cunningham, 1998). Research involving tomato-basil companion planting was reported to increase the total yield and biomass per plant compared to monoculture of tomato (Bomford, 2009; Carvalho et al., 2012). However, the reason regarding this growth and yield increase phenomenon is still unclear. This positive phenomenon may not only involve pest control effect as claimed by gardeners, but also through some phytochemical changes in inner biochemical status of tomato due to the companionship. Furthermore, whether this phenomenon is exclusive to tomato-basil companionship or other *Lamiaceae* herbs shows the same effect is still unclear. As such, the present experiment was conducted to evaluate the impact of some *Lamiaceae* herbs companion planting on tomato growth and changes in the secondary metabolites. Furthermore, the free amino acid changes in tomato due to companion planting was also evaluated using tomato-basil companionship as a model.

Materials and Methods

Growing of tomato and herb seedlings and setup of companion system: Seeds of basil (*Ocimum basilicum* L.), peppermint (*Mentha piperita* L.), hyssop (*Hyssopus officinalis* L.) and tomato (*Solanum lycopersicum*, cv. Momotaro 8) were used in the experiment. Tomato-basil companion planting was used as base model as it is the most common example of companion system given by the garden producers. The other herbs were used to see whether this growth regulation effect was exclusive tomato-basil companion or other herbs of the same family could show similar results. Seeds of the herbs and tomato were sown in 72-hole seed tray (280 mm×545 mm×49 mm) filled with autoclaved commercial soil (Supermix A; Sakata Seed Corporation, Yokohama, Japan). Four weeks after sowing, the seedlings were transplanted to pots (150 mm×130 mm, 1600 ml) also containing the autoclaved commercial soil. The experimental setups were: 1. Tomato mono culture (C), 2. Tomato + Basil (1:1), 3. Tomato +

Basil (1:4), 4. Tomato + Basil (1:8), 5. Tomato + Basil with mesh (1:1m), 6. Tomato + Basil with mesh (1:4m), 7. Tomato + Basil with mesh (1:8m), 8. Tomato + peppermint (1:1), 9. Tomato + peppermint (1:4), 10. Tomato + peppermint (1:8), 11. Tomato + Hyssop (1:1), 12. Tomato + Hyssop (1:4), 13. Tomato + Hyssop (1:8). The tomato-basil companion setup was used as a model for mesh separation treatment. A non-woven mesh was placed in the pot with the tomato plant in the center; separating tomato roots in all sides from the roots of basil plant/plants transplanted keeping a distance of 4 cm. Ten pots per treatment were grown in greenhouse 30 ± 4/24 ± 4 °C day/night temperature with 12-13 h photoperiod (750-1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and 70-80% relative humidity.

Growth analysis of the tomato plants: Four weeks after transplanting, the plants were uprooted and the roots of the tomato plants were cleaned from soil and debris. Then 4 tomato plants per treatment were taken and separated to shoot and root. The plant parts were dried using a constant temperature drier (ETTAS 600B; AS ONE Corporation, Osaka, Japan) at 80°C for two days. Then the dry weight of shoots and roots were measured. The rest of the plants were cryopreserved for further analysis. Based on the results of the growth parameter considered, the tomato plants under 1:1 treatment was considered for secondary metabolites analysis.

Analysis of secondary metabolites in tomato: Analysis of secondary metabolites in tomato was conducted according to the method of Vos et al. (2007). From the cryopreserved samples of five plants, 1.2 g of tomato plant parts (leaf, stem and root) were pulverized separately in mortar with liquid nitrogen to give a fine powder and mixed with 6 ml ultrapure water to prepare sample extract solutions. The sample solutions were then centrifuged (13,000 rpm, 4°C, 15 min) and the supernatant were filtered through a sterilizing filter (0.45 µm; ADVANTECH Co. Ltd., Japan). The samples were further centrifuged (13,000 rpm, 4°C, 15 min) using Nanosep 10K (Nihon Pall Ltd. Tokyo, Japan) to remove proteins in the extract.

The samples were analyzed 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 thermostation at 40°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 sample injection volume was 7.5 µL. The gradient profile was as follows: 0–6 min, 95% A; 6–12 min, 75% A; 12–30 min, 65% A; 30–32.5 min, 5% A; and 32.5–35 min, 95% A. The mass range of electrospray ionization was analyzed in negative mode at 50-1000 m/z using a mass spectrometer (Xevo Q Tof MS, Waters Corporation, Milford, USA), and the MS/MS collision was performed at 30 V. A mass chromatogram of the m/z value of each component in the extract was prepared from the measurements obtained using the retention time.

Analysis of free amino acid content in Tomato:

Sample preparation: Frozen samples (0.2 g, leaves and roots, excluding lateral roots) were extracted using 3 ml of 0.2 N perchloric acid solution and then centrifuged at 4,000 rpm at 4^{o}C for 10 min. After adjustment to pH 4, the supernatant was again centrifuged at 13,000 rpm at 4^{o}C for 5 min. The supernatant was subsequently filtered through a syringe filter for use as an analysis sample (Nimbalkar et al., 2012).

Analytical samples were derivatized using the AccQ·Tag Ultra Derivatization Kit (Waters Corporation, Milford, USA) (Fig. 58). Analytical samples (30 µl) were mixed with 210 µl of borate buffer and 60 µl of derivatization reagent. The reaction mixture was immediately mixed and left for 1 min at room temperature. Subsequently, the solution was incubated at 55^{o}C for 10 min in a water bath. After cooling, the reaction mixture was used for UPLC injection.

Instrumentation and chromatographic condition for UPLC-MS: The ACQUITY UPLC BEH C18 (1.7 µm, 2.1 × 100 mm, Waters Corporation, Milford, USA) reversed phase column

was used under 25°C. The chromatographic condition and gradient used in the experiment showed in Table 4. The mass spectrometer (Xevo Q Tof MS, Waters Corporation, Milford, USA) measured the analysis mass range electro spray ionization in positive mode at 100-1000 m/z. A mass chromatogram (Abs Window 0.05 Da) of the m/z value of each amino acid was prepared from the measurement results, and the amino acid content was measured according to the peak integration value (Table 5). Data analysis was executed using the Waters Masslynx software, USA. Amino acid mixed standard solution H-type (Wako Pure Chemical Industries Ltd., Japan) was used for making a standard curve. Twenty-one kinds of free amino acids were analyzed, all of which were included in the standard solution.

Statistical analysis: The mean values for dry weight of shoots and roots of tomato were analyzed by Tukey's multiple range test at $P < 0.05$. For free amino acid content, the significant differences were determined based on *t* test at $P < 0.05$. All the analyses were conducted using XLSTAT 2012 pro statistical analysis software (Addinsoft Inc. New York, USA).

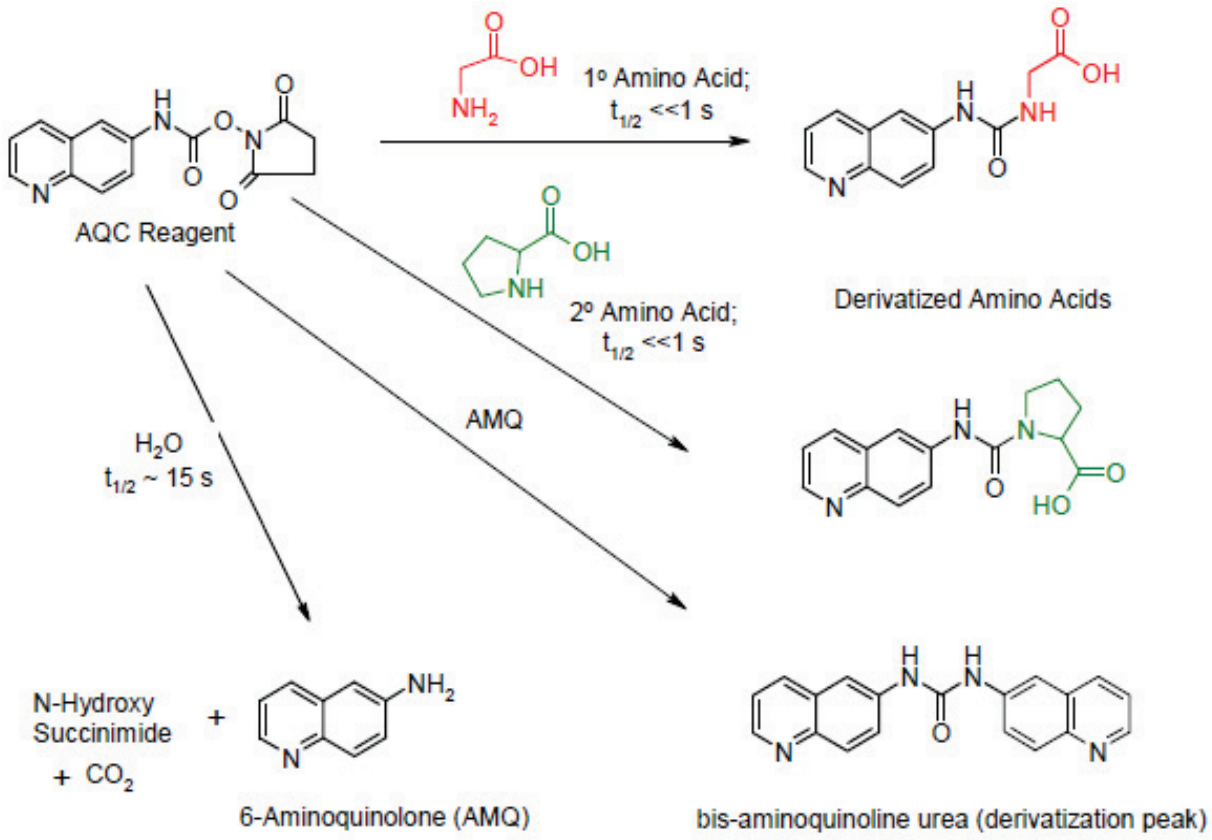

Fig. 58. Amino acid derivatization by AccQ-Tag Ultra Derivatization Kit (Waters Corporation, Milford, USA).

Table 4. Chromatographic conditions.

Column	ACQUITY UPLC BEH C 18 (1.7 µm, 2.1 × 100 mm, reversed phase column, Waters).			
Mobile phase A	0.1% formic acid			
Mobile phase B	acetonitrile			
Injection volume	5 µl			
Gradient	Time (min)	Flow (ml/min)	Mobile phase A (%)	Mobile phase B (%)
	0.0	0.4	99.9	0.1
	12.0	0.4	50.0	50.0
	13.0	0.4	99.9	0.1
	15.0	0.4	99.9	0.1

Table 5. Retention time and m/z value of derivatized amino acids

Amino Acids	Retention time (min.)	m/z (positive)
Histidine	4.39	326.127
Asparagine	4.88	304.095
Arginine	4.98	345.141
Glutamine	5.25	317.098
Serine	5.28	276.259
Glycine	5.48	246.049
Aspartic acid	5.76	304.051
Citrulline	5.77	346.119
Glutamic acid	5.94	318.057
Threonine	6.09	290.069
Alanine	6.41	260.061
γ-aminobutyric acid (GABA)	6.44	274.084
Proline	6.72	286.082
Lysine	7.14	487.173
Cystine	7.19	581.093
Tyrosine	7.83	352.071
Methionine	7.96	320.059
Valine	7.98	288.077
Isoleucine	8.80	302.089
Leucine	8.89	302.089
Phenylalanine	9.15	336.072

Results

Four weeks after transplanting, the dry weights of tomato shoots and roots under companion planting with basil, peppermint and hyssop showed varying degree of change compared to control (Fig**.** 59 and Fig. 60). The highest dry weights in shoots were observed in plants under 1:1 companion treatment compared to control in all the cases. Even in the case of mesh separation in tomato-basil companion system, the highest dry weight of shoots was observed in 1:1 combination which also showed statistical similarity 1:1 without mesh. The dry weights of roots except for hyssop companionship, did not show any significant difference with control. However, growth restriction was observed in the higher density companion treatments especially in 1:8 treatment. In these cases, the dry wights were found to be lower than that of the plants under control except in peppermint.

From the analysis of secondary metabolites in tomato plants, the major compounds that were identified in tomato plants were shikimic acid and apigenin. The content of both these compounds were found to have increased in tomato stem when grown in association with basil (Table 6). However, no significant increase observed in case of leaves and roots of tomato plants under basil companion planting. On the other hand, in both peppermint and hyssop companionship, the content of both shikimic acid and apigenin increased in all parts of tomato plants.

Regarding the changes in free amino acid contents of tomato due to basil companionship, several amino acid contents changed significantly in leaves and shoots of plants under 1:1 treatment compared to control. In case of leaves, significant increase was observed in phenyl-alanine, iso-leucine, valine, alanine, lysine and GABA contents in plants under 1:1 treatment compared to control (Fig. 61). In contrast, higher content of valine, alanine, proline, GABA, serine and glutamine was observed in the stems of plants under 1:1 companion treatment

compared to control (Fig. 62). In both cases, the amino acids other than the ones mentioned in leaves and shoots respectively did not express any significant increase or decrease. However, in case of roots, no significant difference was observed in any free amino acid contents between the treatment considered and control (Fig. 63).

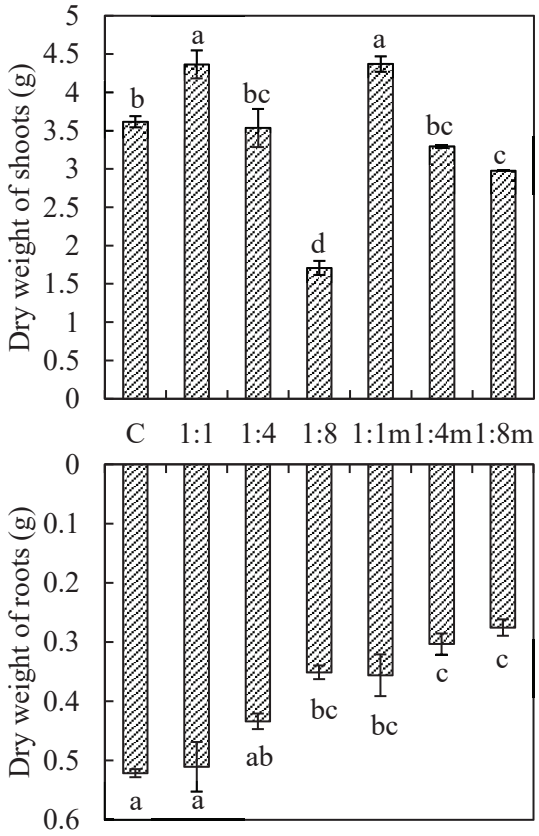

Fig. 59. Dry weight of shoots and roots of tomato plants with basil companion planting. C, single tomato; 1:1, tomato+basil (1:1); 1:4; tomato+basil (1:4); 1:8, tomato+basil (1:8); 1:1m, tomato+basil with mesh (1:1), 1:4m, tomato+basil with mesh (1:4); 1:8m, tomato+basil with mesh (1:8). Columns denoted by different letters indicate significant difference according to Tukey's test ($P < 0.05$).

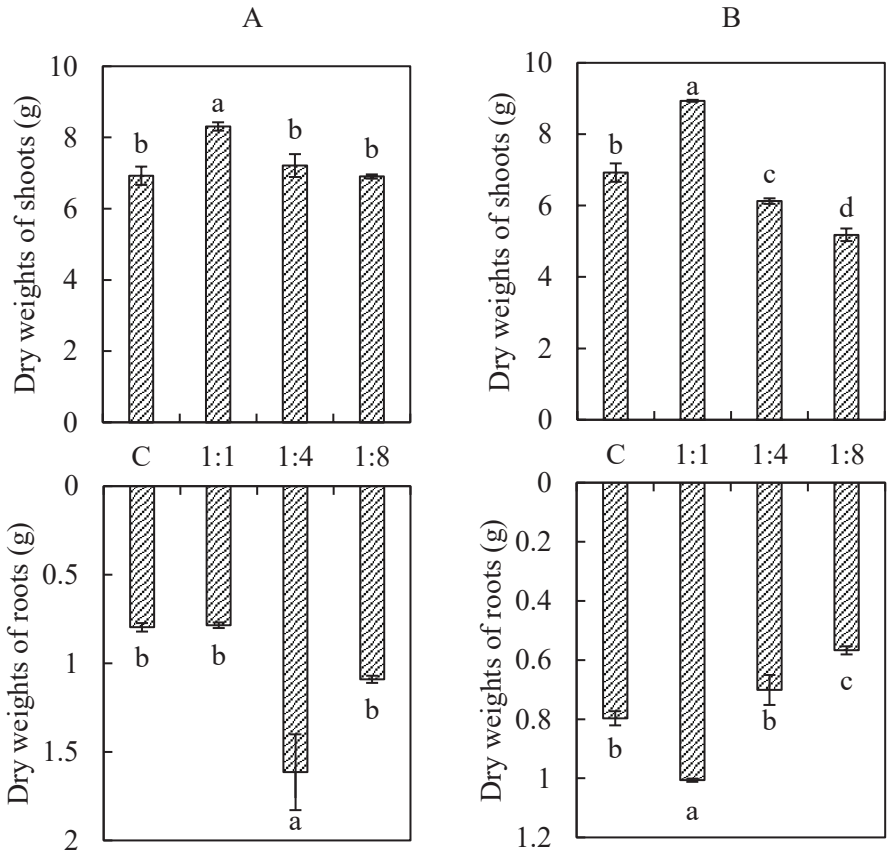

Fig. 60. Dry weight of shoots and roots of tomato plants with peppermint (A) and hyssop (B) companion planting. C, single tomato; 1:1, tomato+peppermint/hyssop (1:1); 1:4; tomato+ peppermint/hyssop (1:4); 1:8, tomato+ peppermint/hyssop (1:8). Columns denoted by different letters indicate significant difference according to Tukey's test ($P < 0.05$).

Table 6. Influence of companion plants on changes in secondary metabolite content in tomato.

Companion plant	Tomato plant part	Shikimic acid	Apigenin
Basil	Leaves	–	–
	Stem	○	○
	Roots	–	–
Peppermint	Leaves	○	○
	Stem	○	○
	Roots	○	○
Hyssop	Leaves	○	○
	Stem	○	○
	Roots	○	○

Here, –, no change; ○, increased.

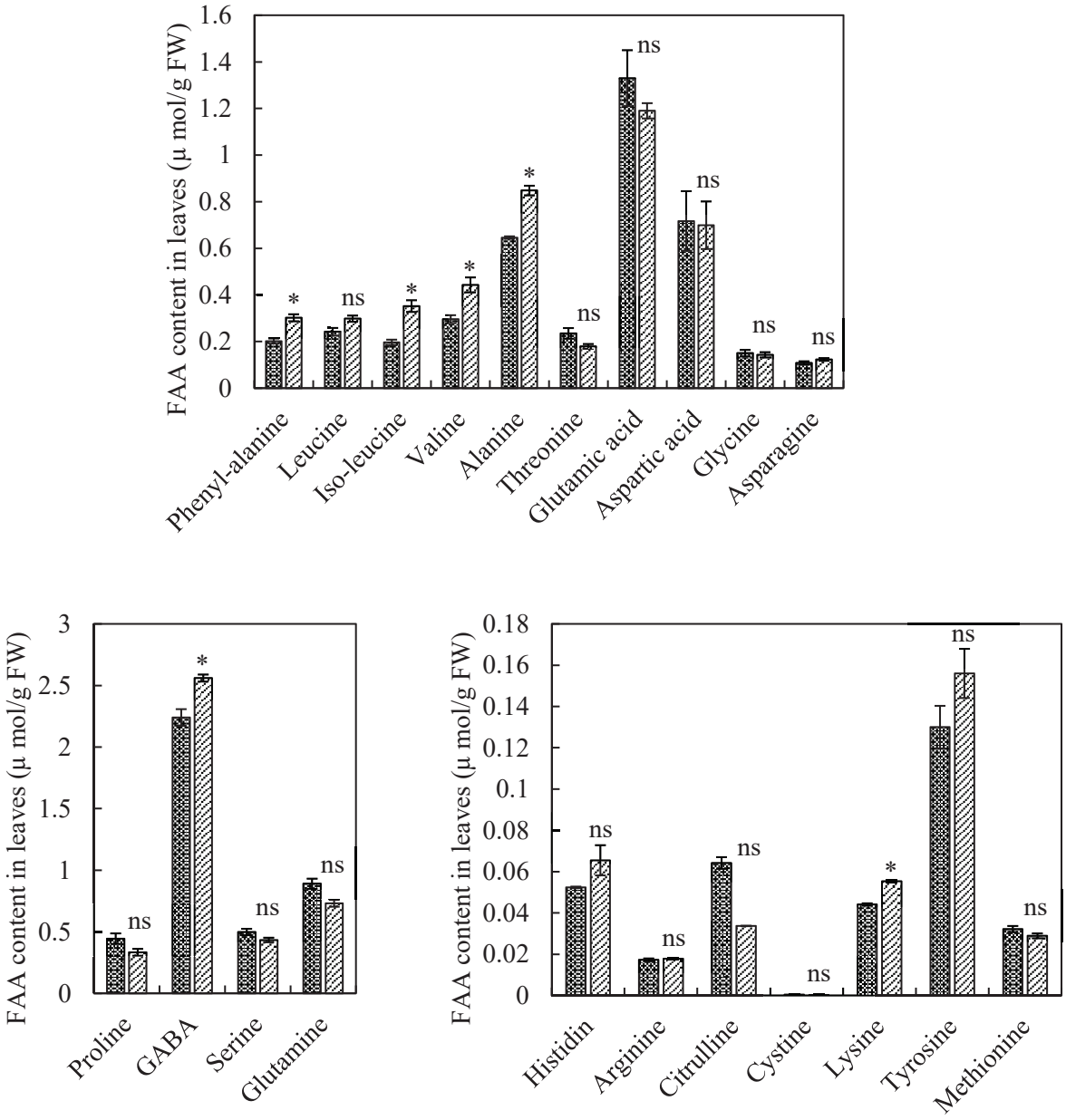

Fig. 61. Free amino acid (FAA) content in leaves of tomato plants grown with basil companion planting. Here, ▩, Tomato mono; ▨, Tomato + Basil (1:1). *, significantly different according to *t*-test ($P < 0.05$); ns, non-significant.

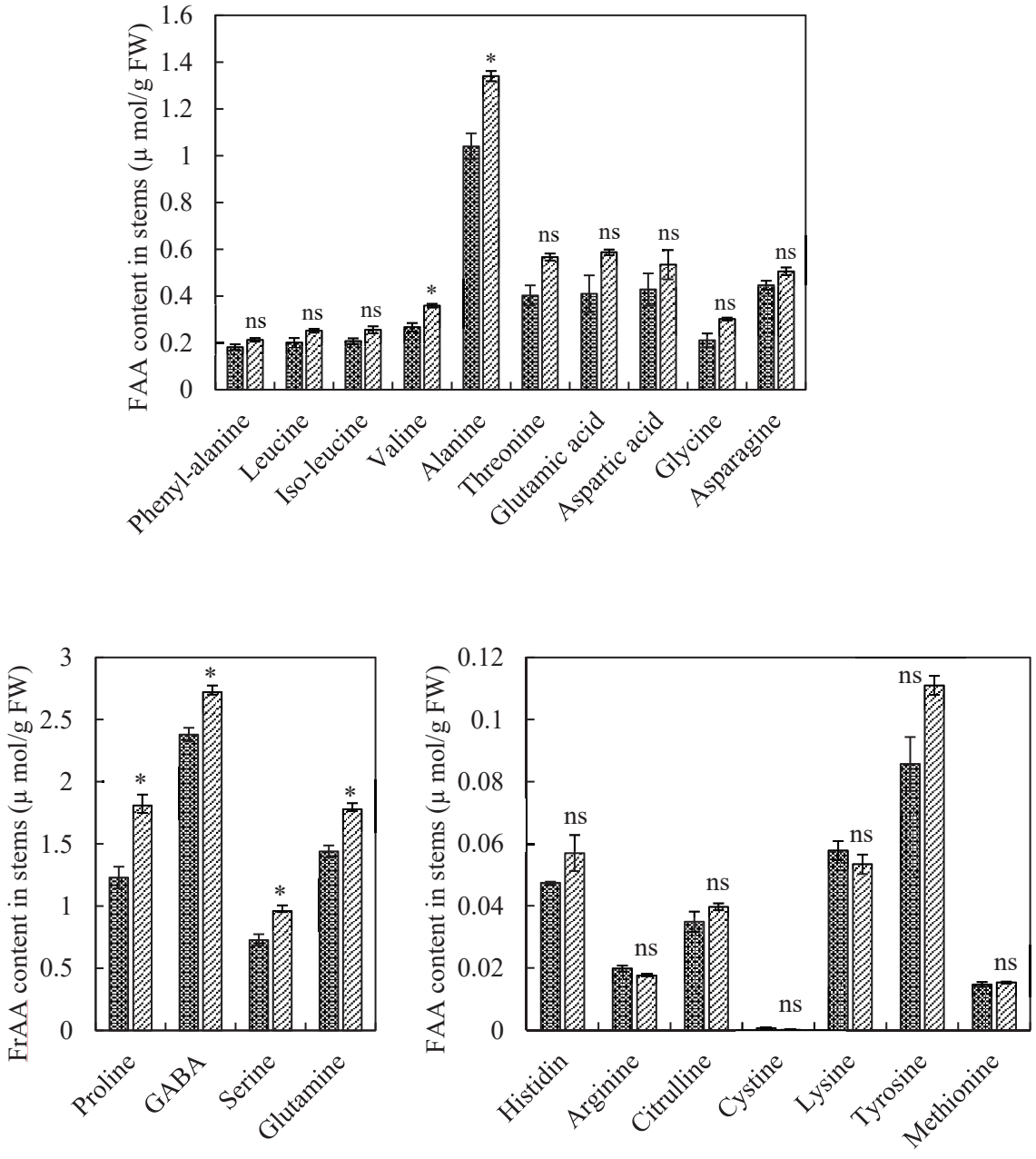

Fig. 62. Free amino acid (FAA) content in stems of tomato plants grown with basil companion planting. Here, ▩, Tomato mono; ▨, Tomato + Basil (1:1). *, significantly different according to *t*-test ($P < 0.05$); ns, non-significant.

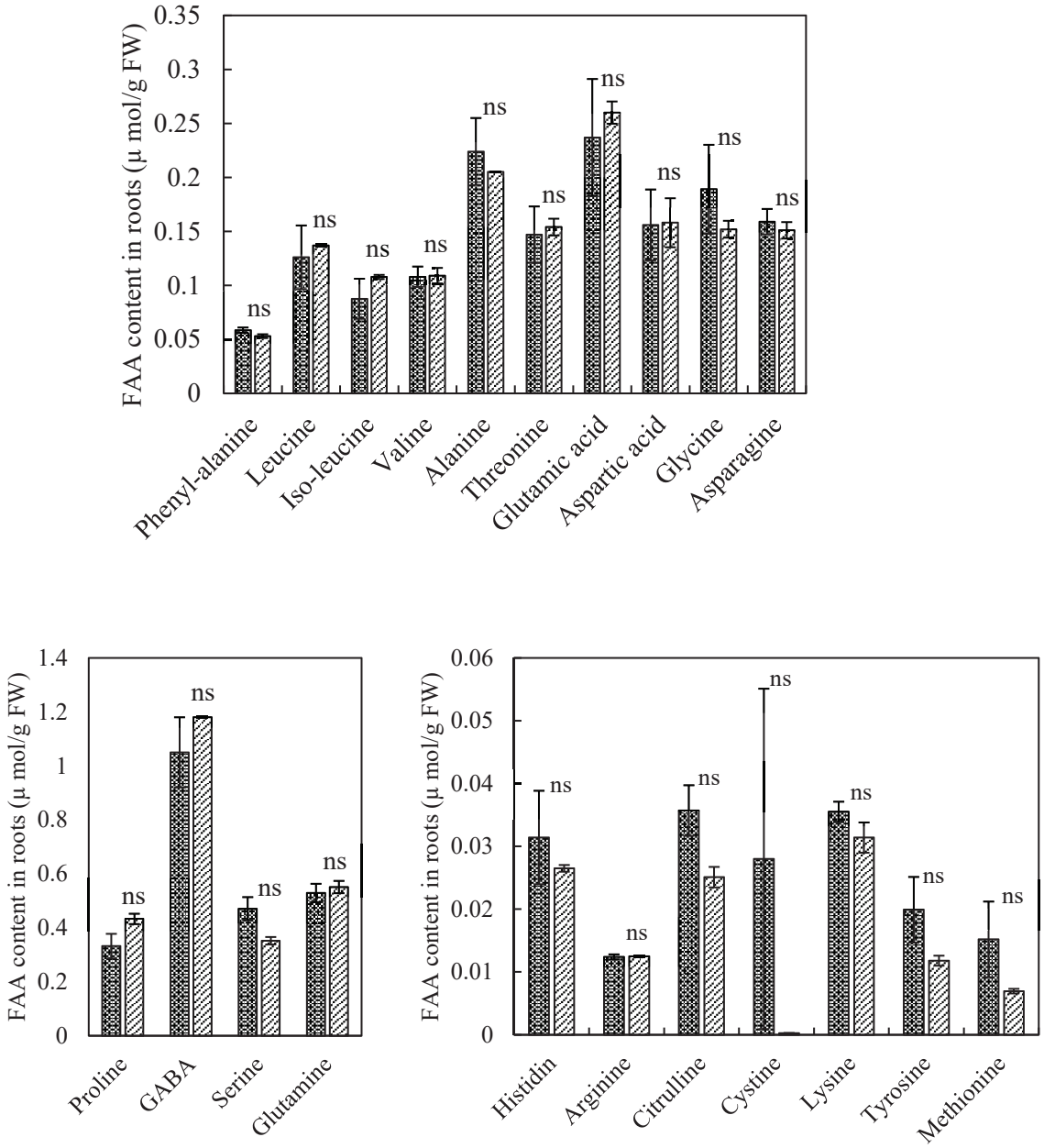

Fig. 63. Free amino acid (FAA) content in roots of tomato plants grown with basil companion planting. Here, ▩, Tomato mono; ▨, Tomato + Basil (1:1). *, significantly different according to *t*-test ($P < 0.05$); ns, non-significant.

Table 7. Influence of companion planting on herb growth, tomato dry weight indices and aroma quality of the herbs.

Parameters	Basil	Peppermint	Hyssop
Herb growth	High	Medium	Low
Index of tomato shoot dry weights*	120	119	129
Aroma quality	High	High	Low

*Calculated as: Shoot dry weight index $= \frac{\text{Dry weight of plants under 1:1 companionship}}{\text{Dry weight of plants in mono culture}} \times 100$

Discussion

In the present study, tomato plants grown with basil companionship showed a varying degree of growth expression depending on the plant density. The 1:1 combination appeared to have growth boosting ability compared to control. This may be attributed to the less competitive behavior from basil in the companion system as reported by (Bomford, 2009). According to the report, basil plants tend to be subordinate crop in companion crop system. This although resulting in growth retardation of basil, do not interfere with the growth of the major or dominant crop. As such, the competition between tomato and basil plants in 1:1 system for nutrition, water, light and space was lower that contributed to the growth of tomato plants. Furthermore, this positive phenomenon of companionship on tomato is not limited to only basil as observed from the present study. Herbs of *Lamiaceae* family like peppermint and hyssop seemed to have the similar growth promoting influence on tomato as evident from the results. However, with the higher density of the herb plants like 1:4 or 1:8, the competition pressure accumulated and thus hampered the growth of tomato plants as observed in the result. Trials with other crops under companion planting system also exhibited better growth with efficient use of the net input available, turning up better yields (Feike et al., 2010). Miyazawa et al. (2010) had also found similar growth and yield improvement in crops grown together than individually and attributed these phenomena to better utilization of available resources by the plants.

The major components identified in tomato plants through UPLC-MS analysis were shikimic acid and apigenin, and their content in tomato shoot was influenced significantly in plants grown with herb companionship. It has been reported that, intercropping pressure has the ability to influence the accumulation of minerals and other secondary metabolites in plants increasing their quality (Inal et al., 2007; Li et al., 2014; Tong et al., 2015). Similar phenomenon must have occurred in the present experiment. The growth regulation in tomato

plants by shikmic acid was reported to be mediated through increasing longevity of leaves by retaining chlorophylls and increasing mineral contents (Al-Amri, 2013). Furthermore, shikimic acid has been related to the increased contents of soluble sugar and lycopene which influences the taste and appearance of tomato (Favati et al., 2009; Al-Amri, 2013). This could possibly explain the claim of the garden producers about the improvement of taste of tomato grown in herb companionship. On the other hand, flavonoids like apigenin plays several important roles in plant growth and development like increasing tolerance to biotic and abiotic stress, hormone transportation, acting as phytoalexins etc. As such, an increase in these compounds is desirable in plants to maintain as well as boost the growth and subsequently the yield and quality of a crop.

Regarding free amino acids, a significant increase was observed in several of them in both leaves and stems of tomato plants. The influence of basil companion planting on increased shikimic acid production may have played a role in the increase in several amino acids; as shikimate pathway is used by plants for biosynthesis of aromatic amino acids (Santos-Sánchez et al., 2019). Amino acids play several roles in plant life such as stress management, hormone precursors and regulation (Zhao, 2010; Maeda and Dudareva, 2012). Beside these, another important role played by the amino acids is the regulation of several physiological processes in plants like nutrient uptake specially nitrogen, antioxidant metabolism and root development (Miller et al., 2007; Hildebrandt et al., 2015; Weiland et al., 2015). Amino acids like phenylalanine, L-alanine, leucine, lysine and glutamine had been reported to influence glutamate receptors in plants (Forde and Roberts, 2014) that in turn mediate a number of plant responses like changes in root architecture, plant stress signaling, carbon metabolism, stomatal movements, photosynthesis and plant immunity (Weiland et al., 2015). GABA has been reported to act as endogenous signaling molecule in plants that regulates growth and development and protect from various environmental stresses (Ramos-Ruiz et al., 2019).

Proline had been reported to influence in plant growth and development by being an important component of cell wall matrix to combat stress conditions (Kishor and Sreenivasulu, 2014) whereas serine had been reported to participates in the biosynthesis of several biomolecules required for cell proliferation, including amino acids, nitrogenous bases, phospholipids, and sphingolipids (Ros et al., 2014). So, an increase in these important free amino acids in tomato plants possibly played an important role in the growth improvement observed from the experiment.

In the present experiment, the soil used for growing both tomato and herb plants was autoclaved prior seed sowing and subsequent transplanting. This eliminated the possibility of any microorganism influence on the growth improvement in tomato. Furthermore, in the case of mesh separation, no physical contact between the tomato and basil roots were present. As such, no physical stimulation or signaling could be present. So, the only possible way for any influence occurring from the companion planting system was through chemicals ques generated from the herb companions. To further evaluate this hypothesis, the relative growth of the basil, peppermint and hyssop compared to tomato (by visual observation), index of tomato shoot dry weights and aroma (by smell test) were measured (Table 7). From the evaluation it was observed that relative to tomato, the growth of basil, peppermint and hyssop could be graded as high, medium and low respectively. Furthermore, in aroma evaluation, both basil and peppermint gave distinguishable aromatic presence whereas hyssop had a very low aromatic presence. However, the tomato plants under hyssop companionship showed higher shoot dry weight index compared to basil or peppermint. If we consider the volatile compound or essential oils of the herbs to be responsible for growth improvement in tomato, then plants with hyssop companionship should express a lower dry weight index compared to basil and peppermint. As such, the only possible explanation of the growth improvement observed in tomato would be the influence of some root exudates from the herbs. However, further in-depth

evaluation is necessary to clarify it. Identification of such biochemical influencers would enable us to gain the beneficial effect with their direct application in the crop production system and open a new avenue of crop growth promotion.

Chapter 4- Conclusion

The present experiment in this chapter evaluated the growth improvement and changes in secondary metabolites and amino acids in tomato when grown in companionship with *Lamiaceae* herbs. The results indicated a growth improvement in 1:1 combination of tomato and herbs with increase in some important secondary metabolites and amino acids. Increase of these primary and secondary molecules might have played an important role in the growth promotion. The experimental setup ensured that there was no biological and physical intervention present in the system. As such, there might be some chemical cues from the herbs that were grown in companionship that influences these changes in the tomato plants. The present experiment acts as a fundamental research to address the longstanding claim from garden producers about the beneficial impact of such companionship. However, further in-depth analysis is required to clarify the mechanisms involved in the growth and subsequent yield improvement of tomato plants.

Summary

Medicinal plants have long since being used in pharmaceuticals, food, cosmetics industries due to containing many biologically active compounds in them which provides a wide range of beneficial effects. Among them, the plants under *Lamiaceae* family have been extensively studied and are found to contain many important secondary metabolites and other active compounds which enabled their use in cosmetics, flavoring, fragrance, perfumery and pharmaceutical industries. However, importance of using these herbs and their extracts in controlling diseases of plants is a less ventured area. Furthermore, the reports that are currently available on these herbs for phytopathogen control focus primarily on the essential oils and other organic extraction compounds. As such, transition of the disease suppression effect observed by initial *in vitro* analysis is quite difficult due to the innate volatile nature of the essential oils and organic compounds. On the other hand, information regarding the use of the water-soluble secondary metabolites of these herbs in growth promotion and disease suppression of important crops is unclear which can be potential avenue to pursue. In this study, we aimed to evaluate the potential of *Lamiaceae* herbs water extracts in growth promotion and disease suppression of the important vegetable crops with identification of the secondary metabolites by metabolomic analysis.

Ten herbs under the *Lamiaceae* family namely oregano (*Origanum vulgare* L.), catnip (*Nepeta cataria* L.), sage (*Salvia officinalis* L.), dark opal (*Ocimum* spp.), thyme (*Thymus vulgaris* L.), basil (*Ocimum basilicum* L.), hyssop (*Hyssopus officinalis* L.), peppermint (*Mentha piperita* L.), lamb's ear (*Stachys byzantina* K.) and lemon balm (*Melissa officinalis* L.) were investigated to determine their antioxidative capacity with antifungal activity against several *Fusarium* species *in vitro*. From the results of the investigation, among the 10 herbs, oregano, sage, hyssop and lemon balm expressed higher DPPH radical scavenging activity, total polyphenol and ascorbic acid content compared to others. Furthermore, the *in vitro*

investigation of distilled water extracts (0.5% and 2%) of the herbs against the *Fusarium* species major expressed suppressive activity from the aforementioned 4 herbs. On the basis of this investigation, these 4 herbs were selected for bioassay against several diseases of important horticultural crops. The results for the bioassay showed that these herbs water extracts had the ability to reduce the incidence of Fusarium wilt in strawberry, cyclamen and Fusarium root rot in asparagus. Furthermore, herb water extracts especially lemon balm suppressed anthracnose of strawberry also envisioning a dual suppressive ability.

To identify the secondary metabolites responsible for the activities of these 4 herbs, a metabolomic analysis of oregano, sage, hyssop and lemon balm was performed by UPLC-MS/MS (Q Tof). Through the fragment analysis done by MS/MS, presence of rosmarinic acid and caffeic acid were confirmed in the herb extracts and they expressed inhibitory effect against the disease-causing organism in *in vitro* evaluation. So, the presence of these important and active secondary metabolites was found to be responsible for the disease suppression activity of the herb extracts. Beside these phenolic acids, presence of luteolin in lemon balm, apigenin and protocatechuic acid in oregano were also confirmed. Therefore, different herbs water extracts may contain other important secondary compounds in them besides the common phenolic acids and they may act synergistically in disease suppression when the herb extract is used. As such, improvement of quality of the herbs and content of these secondary metabolites are important for attaining more beneficial effect. With this view, we used arbuscular mycorrhizal fungi as a way to improve the growth and quality of these 4 herbs. From the results of the experiment, it was found that, treatment with arbuscular mycorrhizal fungi boosted the growth and foliage of the herbs. Colonization occurred in all the herbs under treatment with a difference in degree between the herb species. In addition, increased content of several important secondary metabolites was observed in the mycorrhizal herbs along with the presence of new secondary compounds compared to non-mycorrhizal herbs. Moreover, to

evaluate the synergistic effect of herb water extract and mycorrhizal fungi colonization on induction of disease tolerance in crops, an experiment was conducted using lemon balm and mycorrhizal fungi against anthracnose of strawberry. Induction of disease resistance was observed in plants under single treatment of herb and mycorrhizal fungi which was boosted further by combined use. The increased antioxidative properties of the strawberry plants under combined treatment was considered responsible in tackling pathogenesis related reactive oxygen species suppression resulting reduced damage compared to control.

To address the long-standing claim of beneficial effect of herb companion planting with major crop, several *Lamiaceae* herbs were selected for evaluation with tomato. Sterilized soil was used in the experiment to eliminate any biological intervention and root zone separation using net mesh was employed to eliminate any physical influence using tomato-basil system as a model. From the results of the experiment, it became clear that, 1:1 combination of tomato-herb had the growth promoting effect on tomato plants regardless of the herbs used as companion, as evidence from the dry weight evaluation. Furthermore, through metabolomic analysis, increase in important secondary metabolites in tomato such as shikimic acid and apigenin was observed in 1:1 combination with herbs compared to control. Regarding primary metabolites like free amino acids, increase in phenyl-alanine, iso-leucine, valine, lysine, GABA, serine and glutamine were observed in different parts of tomato plants under 1:1 combination. From these findings, it was clarified that companion planting with herbs had a growth promoting effect on tomato and this phenomenon was not specific to only tomato-basil companionship contrary to popular belief. The experimental settings also suggested that this positive effect could be result of some chemical cues from the herb extracts that might have influenced several secondary metabolites and free amino acids regulating the growth of the tomato plants.

In short, through this study, we clarified the antioxidative and antimicrobial activity of the several *Lamiaceae* herbs against some important diseases of vegetables focusing on the secondary metabolites found in their water extracts. The findings gave us a simple, eco-friendly and sustainable way of controlling these important diseases. In addition, we obtained fundamental knowledge about the induction of disease resistance and growth promoting effect of such herb's association with vegetable crops.

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