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ABSTRACT

The nitrogen-fixing actinomycete *Frankia* coexists with actinorhizal plants via nodules and supplies nitrogen compounds to the plants. Although communication has been suggested to exist through chemical substances in this nodule symbiosis, the details underlying this mechanism remain elusive. The biphenyl-type diarylheptanoids (BP-CDHs), alnusonol, and alnusdione, previously isolated from the actinorhizal plant *A. sieboldiana* branch wood, are secondary metabolites that accumulate in a limited number of plant species. However, since relatively widely distributed in actinorhizal plants, we investigated whether adding *A. sieboldiana* root extracts and these BP-CDHs could affect plant seedlings inoculated with *Frankia*. The results showed that the addition of root extract or alnusonol significantly increased the number of nodules and lobes more than two times compared with that upon *Frankia* supplementation only. We also proved that the extracted components of this plant affected nodule symbiosis. Finally, we confirmed through LC–MS that the root extract component contained BP-CDH, alnusonol. The above-described results indicate that BP-CDHs, at leaset alnusonol, might function as signal compounds from the plant side of the actinorhizal symbiosis between *A. sieboldiana* and *Frankia*.

Keywords: Actinorhizal plants, Alnusonol, Biphenyl-type diarylheptanoids, Chemical communication, Root nodule formation

Abbreviations: BP-CDH, Biphenyl-type cyclic diarylheptanoid

1. Introduction

Prokaryotes are the only organisms with nitrogen fixation ability. Certain prokayotic species maintain a specific symbiotic relationship (nodule symbiosis) with plants (Franche et al., 2009), e.g., *Rhizobium* (Trinick, 1973, 1979), symbiotic with legumes, and *Frankia* (Dawson, 2008), forming a symbiotic relationship with actinorhizal plants. These are internal symbioses through nodules formed in the plant roots, and plants form a mutualistic symbiosis. This symbiotic process supplies carbon sources to the microorganisms in return for receiving nitrogen sources from them through the root nodules (Baker et al., 1990).

Actinorhizal plants symbiotically form root nodules with and receive ammonia nitrogen from actinomycete *Frankia*, which can fix atmospheric nitrogen (N_2) (Dawson, 2008). These plants play important roles in the terrestrial ecosystems as they increase nitrogen availability, a factor commonly limiting to primary productivity and other ecosystem processes (Hibbs and Cromack, 1990; Karthikeyan et al., 2013).

Alnus sieboldiana (Japanese alder) is a warm temperate actinorhizal plant distributed in upland sites along the Pacific coast in Central Japan (Kitamura and Murata, 1979). This plant grows well on nutrient-poor sites due to coexisting with both mycorrhiza and actinomycetes (Yamanaka et al., 2005). Therefore, *A. sieboldiana* is a pioneer tree in devastated areas after volcano eruptions (Yamanaka and Okabe, 2003, 2006) and has been used for land stabilization and revegetation of slopes and deserted areas (Komukai et al., 2012). We described that *A. sieboldiana* formed nodules after inoculation with a *Frankia* isolate in a previous study establishing techniques for *Frankia* inoculation and cultivation of inoculated plants (Yamanaka et al., 2016).

Research is ongoing on root nodule symbiosis, focusing on the symbiosis of rhizobia and legumes, while its symbiotic mechanisms have been studied in detail (Long,1989). Moreover, another study reported that the symbiosis between legumes and rhizobia was initiated by rhizobia through the recognition of host-specific low-molecular-weight signaling substances, isoflavonoids or flavonoids, by rhizobia (Hassan and Mathesius, 2012, Liu and Murray, 2016, Wang et al., 2018). Subsequently, the rhizobia nodulation genes that recognize legume-secreted flavonoids are activated and biosynthesize the nodulation factor (Nod factor) lipochitooligosaccharide, a responding substance. Finally, when the plant recognizes and accepts the Nod factor, plant roots curl and trap rhizobia to form the nodules (Lerouge et al., 1990). Chemical communication promotes the symbiosis. Actinorhizal symbiosis is also considered to proceed through chemical communication, similar to the symbiosis between legumes and rhizobia. Nevertheless, the chemical communication mechanism during actinorhizal–*Frankia* symbiosis is poorly understood.

In contrast, we examined the extracted *A. sieboldiana* components (Chiba et al., 2013) and described that this plant accumulated BP-CDHs, alnusonol, alnusdione, and alnusone (Fig. 1). Only limited number of plant families, such as *Alnus* (Nomura and Tokoroyama, 1974), *Myrica* (Begley et

al., 1971), and *Casuarina* (Kaneda et al., 1990) are known to biosynthesize these BP-CDHs (Lv and She, 2010, 2012; Jahng and Park, 2018). Interestingly, a high degree of commonality exists between plants that accumulate BP-CDHs and actinorhizal plants forming root nodules with *Frankia*. Moreover, these BP-CDHs can potentially act as signal compounds for root nodule formation, thereby promoting actinorhizal symbiosis.

Therefore, this study investigated how *A*. *sieboldiana* BP-CDH, alnusonol, and alnusdione, or methanol extract supplementation, together with *Frankia*, in *A*. *sieboldiana* seedlings, affected seedling growth, including nodule and lobe formation. Furthermore, we confirmed the presence of the BP-CDHs through LC–MS analyses of the *A*. *sieboldian*a root methanol extract.

2. Results and Discussion

2.1. Effects of root extract, alnusonol, and alnusdione on A. sieboldiana growth and nodulation

Three weeks after *Frankia* inoculation, the growth of the seedlings inoculated with *Frankia* alone (entry 1) was better than that of the non-inoculated controls. However, the growth of seedlings supplemented with the root extract (entry 2), alnusonol (entry 3), or alnusdione (entry 4) was similar to that of the seedlings inoculated with *Frankia* alone (entry 1) (Fig. 2a).

Table 1 summarizes growth results 6 weeks after inoculation. As observed, although the shoot length did not differ between the entries (Fig. 2b), roots were longer in *Frankia*-inoculated seedlings (entries 1–4) than in the controls not inoculated with *Frankia*. Additionally, in seedlings inoculated with *Frankia* and alnusonol, root length significantly increased compared with *Frankia* alone.

We also investigated the number of nodules and lobes 6 weeks after inoculation (Table 1). Results showed that the nodule and lobe number of plants to which the root extract (entry 2) and alnusonol (entry 3) were added significantly increased compared with entry 1 inoculated with *Frankia* alone (Fig. 3). Regarding the addition of alnusdione (entry 4), an increase in nodule and lobe number of plant was observed, although there were no significant differences.

On the other hand, with respect to fresh weight, the biomass of plants inoculated with extracts and CDHs (entries 2-4) was smaller than that of plant inoculated only with *Frankia* (entry 1), and in entry 3, the weight was significantly lower than that of plant inoculated only with *Frankia*. The reason for this is not clear, but we speculate that in plants where many nodules were produced, plant nutrients and energy may also be used for the growth of *Frankia* in the nodules, and that the infection of the nodules and the growth of the plants may not have been synchronized. We are planning to conduct further experiments to observe the number of nodules and the amount of plant biomass over time, starting from the early infection stage.

Actinorhizal plants comprise mostly trees of 3 orders, 8 families, and more than 200 species. Similar to the rhizobia–legume symbiosis, exchanges of specific signal compounds between *Frankia* and actinorhizal plants have been postulated. Recently, several studies have been published to identify the symbiotic mechanism of actinorhizal plants. The genes identified to be involved in legume–rhizobia symbiosis include SymRK (symbiosis receptor kinase, Gherbi et al., 2008), CCaMK (calcium and calmodulin-dependent protein kinase, Svistoonoff et al., 2013), and CgNIN (*Casuarina glauca* NODULE INCEPTION, Clavijo et al., 2015). These genes were confirmed for the actinorhizal plant's symbiosis. Furthermore, auxin released by plants was suggested to act as a negative regulator of nodule formation to balance the benefits of plants and *Frankia* (Champion et al., 2015). However, the root hair deformation factor (a signal substance secreted by *Frankia*), which

a different substance from the Nod factor (lipooligosaccharide) secreted by rhizobia, is involved in symbiosis (Chabaud et al., 2016). Besides, regarding the signal compounds from the plant side, since the number of nodules decreased due to chalcone synthase (CHS) gene RNA interference, flavonoids were proposed to be involved in the actinorhizal symbiosis as in legumes (Abdel-Lateif et al., 2013). Nevertheless, although legumes biosynthesize specific flavonoids, especially isoflavonoids, no flavonoid biosynthesis specifically in actinorhizal plants has been reported.

We previously isolated BP-CDHs, alnusonol, alnusdione, and alnusone, from *A. sieboldiana* branch wood (Chiba et al., 2013) (Fig. 1). Interestingly, despite the limited BP-CDH distribution in the plant kingdom (Lv and She, 2010, 2012; Jahng and Park, 2018). *Alnus* (Nomura and Tokoroyama, 1974), *Myrica* (Begley et al., 1971), and *Casuarina* (Kaneda et al., 1990) actinorhizal plants biosynthesized these compounds. Therefore, BP-CDHs were considered signal substances produced from the tree side during the chemical communication between *A. sieboldiana* and *Frankia*. The effect on nodule formation was clear, and the addition of root extract or alnusonol increased both the nodule and lobe numbers by 2.4-fold compared with entry 1 (supplemented with only *Frankia*).

2.2.A. sieboldiana root extract LC–MS analysis and the confirmation of BP-CDH's presence

Next, we analyzed the root extracts using LC–MS. Fig. 4a shows the total ion chromatogram of the root extracts and alnusonol, as well as the mass chromatogram of the molecular ion of alnusonol (MH^+, *m/z* 311). Fig. 4b presents the mass spectra of each peak. Our results indicate that the root extracts contained alnusonol. However, for alnusdione, under the analytical conditions of this analysis, we detected no trace of this compound peak, even in the authentic sample, and the presence of alnusdione remained unconfirmed in the root extract.

LC–MS analysis of this fraction was conducted to determine the presence of alnusonol and alnusdione in the root methanol extract. We could detect an alnusonol peak with the same retention volume as the authentic compound in the root extract, and the mass spectrum also matched. Alnusonol most probably acts as a signal substance involved in root nodule symbiosis with *Frankia*. However, alnusdione, including the authentic compound, could not be confirmed under these analytical conditions. We considered that alnusdione is unstable with a 1,3-diketon structure, performing keto-enol tautomerism. However, structurally, alnusonol and alnusidione differ in their substituent at C-11, corresponding to either an alcoholic hydroxyl or a carbonyl group. Therefore, they may also be present in the root extract.

Curcumin in *Curcuma longa* was previously investigated regarding the biosynthetic mechanism of linear diarylheptanoid (Katsuyama et al., 2009). During curcumin biosynthesis, a

diarylheptanoid skeleton is formed by condensing two cinnamoyl CoA ester derivative molecules with malonyl CoA using two plant type III polyketide synthase (PKS III) enzymes.

As mentioned above, silencing the gene, which is believed to be the CHS of *Casuariana glauca* reduced the number of *Frankia* nodules (Abdel-Lateif et al., 2013). Further, it has been reported that flavonoids isolated from *Myrica gale* seeds induced the nitrogen fixation of *Frankia* (Popovici et al., 2010). Therefore, flavonoids are considered as potential signal compounds in actinorhizal–*Frankia* and during legume–rhizobia symbiosis. However, as both the flavonoid and diarylheptanoid skeleton condensing enzymes (CHS and diarylheptanoid synthase, respectively) belong to PKS III, it would be necessary to elucidate flavonoids and BP-CDH biosynthetic genes, and enzymes in actinorhizal plants. Therefore, we are attempting to obtain the BP-CDH biosynthetic gene using transcriptome analysis of *A. sieboldiana*. The elucidation of enzymes and genes for the diarylheptanoid skeleton formation is proposed to reveal BP-CDH as a signaling substance for actinorhizal plants and *Frankia*. Furthermore, these compounds may be strongly involved in the expression of root hair deformation factors that are considered to be released from *Frankia*. Thus, further studies are warranted to fill these knowledge gaps.

3. Experimental

3.1. *Frankia isolate preparation*

The *Frankia* isolate (AS-2; Yamanaka et al., 2003, 2016) used in this study was obtained from *A. sieboldiana* root nodules. The isolate was cultured in a nitrogen-free BAP liquid medium (Murry et al., 1984) in the dark at 30°C for 2 weeks. Subsequently, the cultured *Frankia* culture medium was homogenized with a pencil mixer dx (AS ONE, Osaka, Japan) and centrifuged at 2,300 *g* for 15 min. The collected *Frankia* isolate was later washed once with sterile water and centrifuged, after which its cell volume (pcv) was measured. A 20-µL pcv cell suspension containing sterilized water equivalent to 50 times the cell volume, was used in subsequent experiments.

3.2. *The A. sieboldiana root extract and cyclic diarylheptanoid preparation*

A. sieboldiana root samples were collected from the campus of Shizuoka University, air-dried, then powdered. The obtained root powder was sieved (40–100 mesh) and extracted with methanol using a Soxhlet extractor for 8 h, after which the extract was concentrated to dryness. This methanol extract was used as an *A. sieboldiana* root extract.

Additionally, we used *A. sieboldiana*-extracted alnusonol and alnusdione as reported by Chiba et al. (2013) as BP-CDHs in this experiment.

Finally, the root extract and BP-CDHs were dissolved in ethanol to a concentration of 400 µg/mL and for use in subsequent experiments.

3.3. A. sieboldiana seedling preparation

A. sieboldiana seeds, from the stock of the Forestry and Forest Products Research Institute, were used and soaked in running water for several days. The seeds were stirred in 0.5% (w/v) benomyl solution (Benlate wettable powder; Sumitomo Chemical Garden Products Inc., Japan) for 1 h. Next, they were transferred aseptically onto a 0.9% agar medium after removing excess water with a Kimwipe. Next, the seeded agar medium was placed in a growth chamber to germinate seeds under the following conditions: 14 h, 25°C, 115.5 µmol·m^{-2}·s^{-1} at day and 10 h, 20°C, at night. After germination, uncontaminated seedlings were transplanted into sterilized soil mixed with soil for turf and perlite (1:1, v/v) in Ray Leach tubes (164 mL; SC-10; Stuewe & Sons Inc., Tangent, OR, USA). The seedlings were then placed into the growth chamber under the above-described conditions and grown by watering every 2 days. After 1 week of growth, the seedlings were supplied with 1/4 strength to complete the Hoagland's solution (Table S1, Arnon and Hoagland, 1940).

One week before *Frankia* inoculation (4-week-old seedlings), the nutrient solution was changed to a 1/4 nitrogen-free Hoagland solution (Table S1), after which inoculation was conducted. After inoculation, the seedlings were supplied with a 1/4 nitrogen-free Hoagland solution once a week.

3.4. Frankia inoculum and extractive supplementation to the seedling roots

The *A. sieboldiana* seedling roots grown for five weeks were inoculated with 1 mL of the above-described *Frankia* suspension (20-µL pcv). Next, the seedlings were supplemented with 100 µL ethanol solution (400 µg/mL) of the root extract, alnusonol, or alnusdione. Fifteen seedlings were examined in each experiment, grown in the growth chamber described above, watered with 15 mL of water once a day, and supplemented with 15 mL of 1/4 nitrogen-free Hoagland solution once a week. Plants not inoculated with *Frankia* (Control 1, 10 seedlings) and seedlings inoculated with 1 mL of *Frankia* suspension (Control 2, 15 seedlings) were prepared as controls.

3.5. Observation of Frankia's effects and extract supplementation

After 6 weeks, we measured plant shoot and root lengths, including the fresh weight ~~dry mass~~ of seedlings. Then, we counted nodule formation. We also counted the number of nodules and lobes using a stereomicroscope (Wraymer LW-720T).

Subsequently, a one-way analysis of variance (ANOVA) was used to examine the effect of *Frankia* inoculation and extract addition on the growth and nodulation of *A. sieboldiana.* ANOVA showed significant differences, whereas Scheffe's test was used to compare the treatments.

3.6. Root extract LC–MS analyses

The root extract was subjected to mass spectrometry using a Shimadzu LC–MS-2020 system. The HPLC analysis was performed with water: methanol = 50:50 for elution, at a flow rate of 0.5 mL/min, using a Cadenza CD-C18 (Imtakt, Kyoto, Japan), 75 × 4.6 mm, 3 µm as a column. We performed compound detection using a photodiode array. Mass spectrometry was measured by ESI in negative mode (interface voltage: −4.5 kV, interface temperature: 350°C.). Authentic compounds were also analyzed under the same conditions.

Author contributions

SK designed the study and provided funding. ATS, TK, KT, and YY conducted the experiments. TY isolated *Frankia* and organized the *A. sieboldiana* cultivation. ATS, TK, and SK wrote the manuscript. All authors participated in the discussion and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability statement

All data generated or analyzed during the study are included in this published article.

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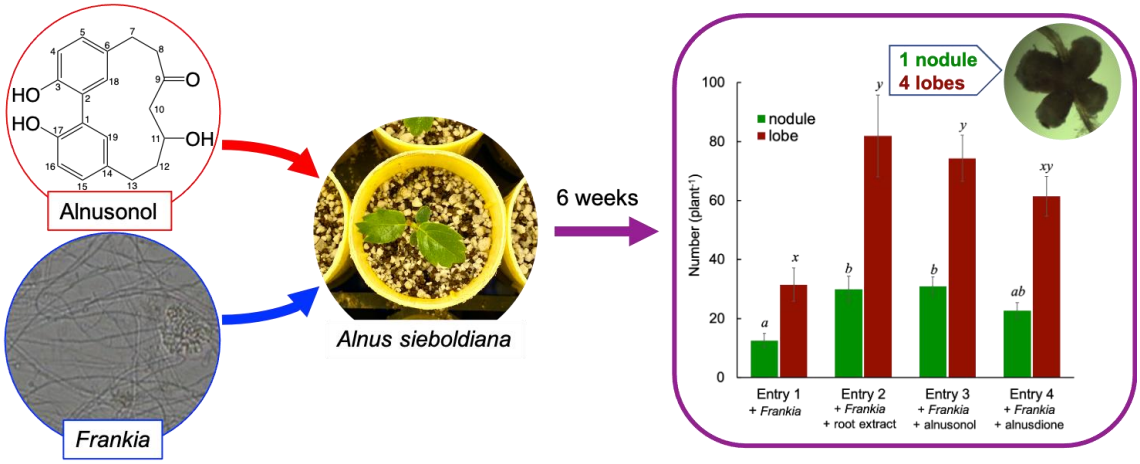
Figure legends

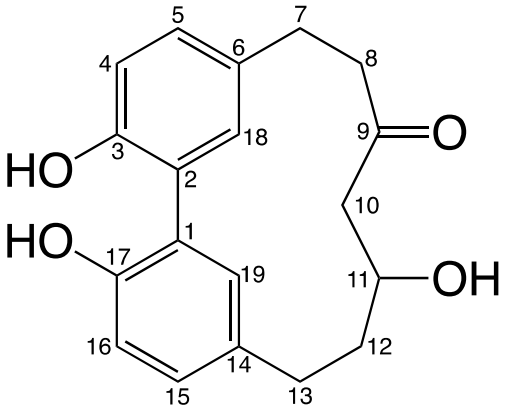
Fig. 1 Chemical structure of the BP-CDHs isolated from *A. sieboldiana*

Fig. 2 *A. sieboldiana* seedling growth after adding *Frankia* and various extracts. **a** Three weeks after *Frankia* inoculation, **b** Six weeks after *Frankia* inoculation. Bars 5 cm

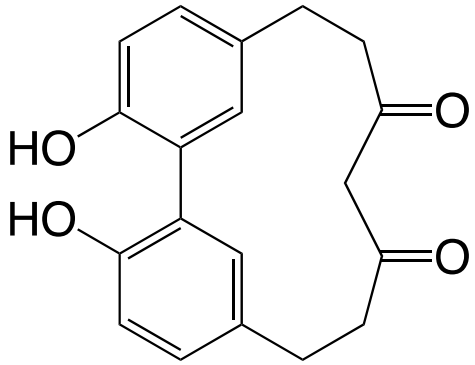
Fig. 3 Effects of extract addition on the number of root nodules (green bar) and lobes (brown bar) of *A. sieboldiana* six weeks after *Frankia* inoculation. Values represent the means ± standard errors calculated from 15 replicates. Different letters (*a* and *x* in nodule and lobe numbers, respectively) indicate significant differences at $P < 0.05$ according to the Scheffe's test.

Fig. 4 LC–MS analyses of the *A. sieboldiana* root extract. **a** Total ion chromatogram (TIC) and mass Chromatogram (MC) (*m/z* 311) of the authentic alnusonol and the root extract, **b** Mass spectra of the authentic alnusonol and root extract peak component at 6.5 min.

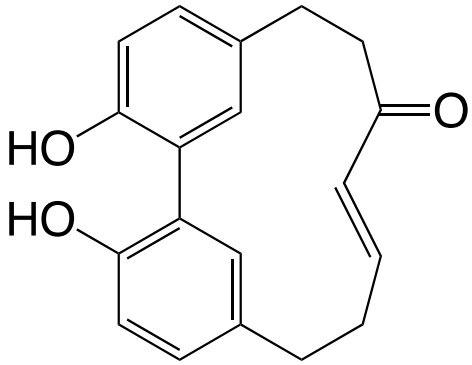

HO
HO
O
OH
Alnusonol
Frankia
Alnus sieboldiana
6 weeks
1 nodule
4 lobes
nodule
lobe
Number (plant-1)
100
80
60
40
20
0
a
x
b
y
b
y
ab
xy
Entry 1
+ Frankia
Entry 2
+ Frankia
+ root extract
Entry 3
+ Frankia
+ alnusonol
Entry 4
+ Frankia
+ alnusdione



Alnusonol



Alnusdione



Alnusone

Fig. 1

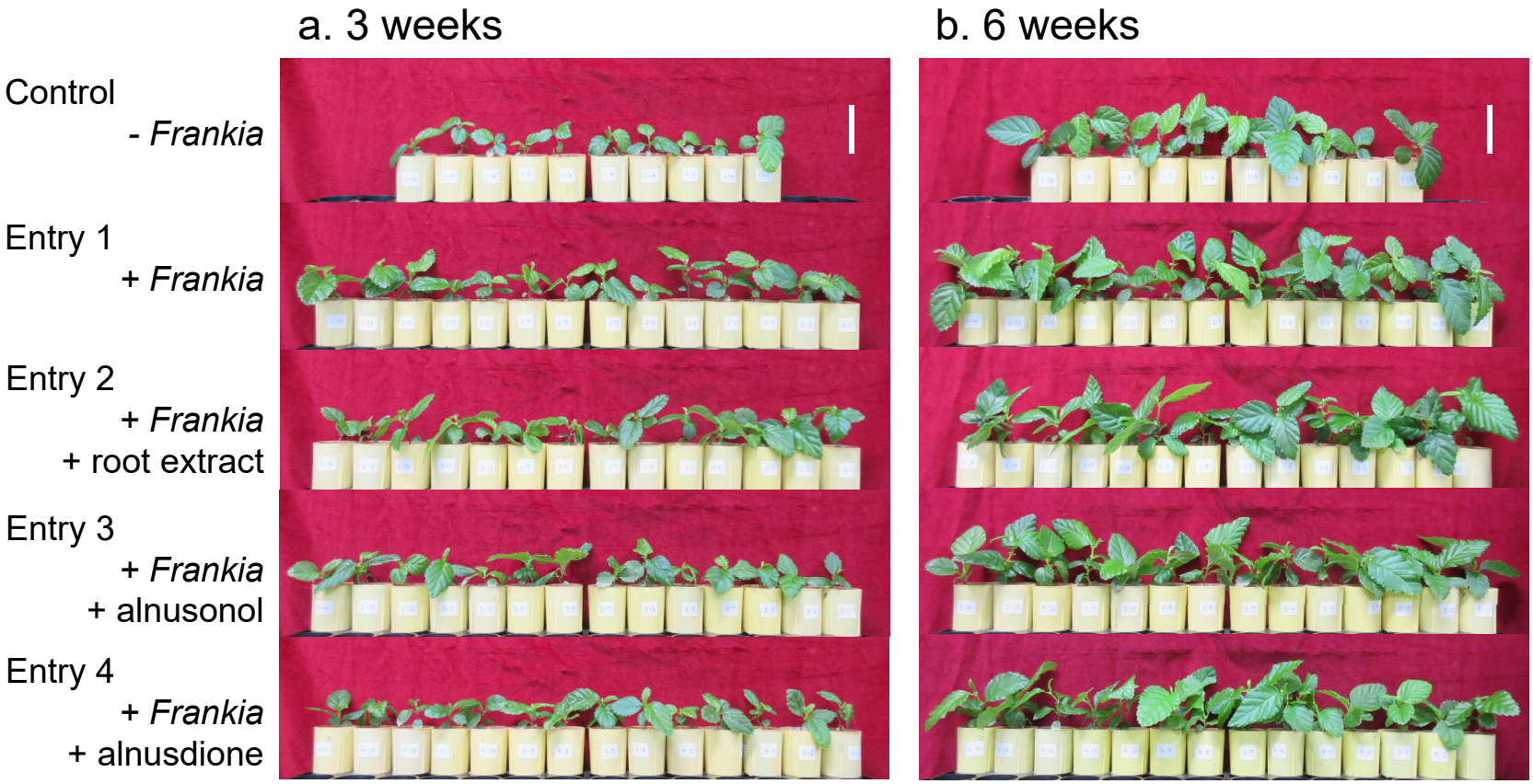

a. 3 weeks
b. 6 weeks
Control
- Frankia
Entry 1
+ Frankia
Entry 2
+ Frankia
+ root extract
Entry 3
+ Frankia
+ alnusonol
Entry 4
+ Frankia
+ alnusdione

Fig. 2

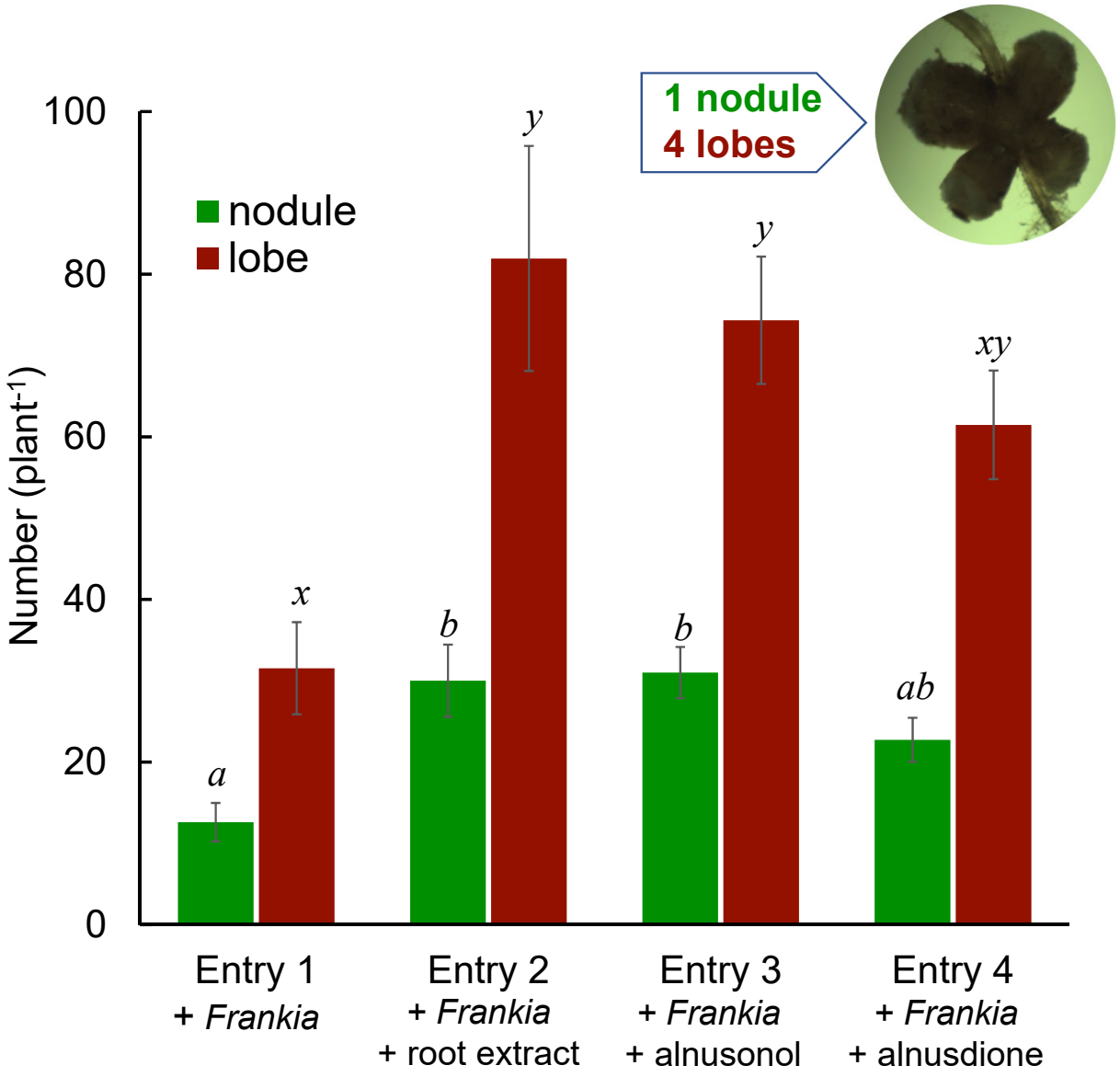

1 nodule
4 lobes
nodule
lobe
Number (plant-1)
100
80
60
40
20
0
a
x
b
y
b
y
ab
xy
Entry 1
+ Frankia
Entry 2
+ Frankia
+ root extract
Entry 3
+ Frankia
+ alnusonol
Entry 4
+ Frankia
+ alnusdione

Fig. 3

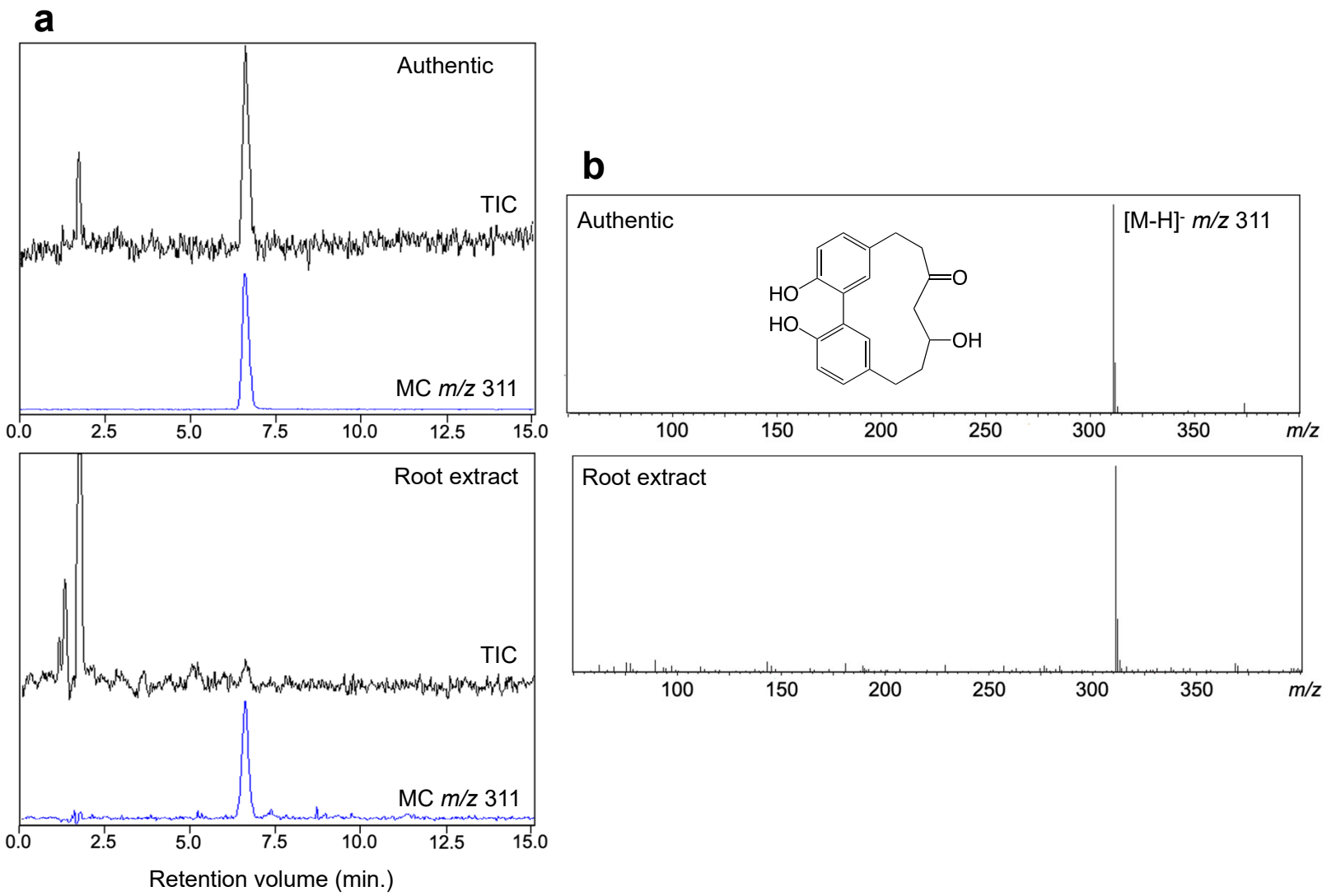

a
Authentic
TIC
MC m/z 311
0.0
2.5
5.0
7.5
10.0
12.5
15.0
Root extract
TIC
MC m/z 311
0.0
2.5
5.0
7.5
10.0
12.5
15.0
Retention volume (min.)
b
Authentic
[M-H]- m/z 311
HO
HO
O
OH
100
150
200
250
300
350
m/z
Root extract
100
150
200
250
300
350
m/z

Fig. 4

Table 1 Growth and nodulation of *Alnus sieboldiana* seedling 6 weeks after inoculation with *Frankia* and various additives.

Entry	*Frankia* inoculum (μL pcv plant^{-1})	Additive	Shoot length (cm/plant)	Root length (cm/plant)	Fresh weight of plant (g)	Noduled plant /tested plant	Nodule Number plant^{-1}	Lobe number	
								plant^{-1}	nodule^{-1}
1	40	none	6.0 ± 0.3	13.1 ± 1.0^{a}	3.1 ± 0.3 ^{a}	15/15	13 ± 2 ^{a}	32 ± 6 ^{a}	2.5 ± 0.2
2	40	root extract	5.7 ± 0.3	14.4 ± 1.1^{ab}	2.4 ± 0.2 ^{ab}	15/15	30 ± 5 ^{b}	82 ± 14 ^{b}	2.8 ± 0.2
3	40	alnusonol	5.6 ± 0.3	17.2 ± 0.7^{ab}	2.0 ± 0.1 ^{b}	15/15	31 ± 3 ^{b}	74 ± 7 ^{b}	2.5 ± 0.1
4	40	alnusdione	6.0 ± 0.3	12.2 ± 0.9^{a}	2.4 ± 0.1 ^{ab}	15/15	23 ± 3 ^{ab}	61 ± 8 ^{ab}	2.8 ± 0.2
Control	none	none	5.0 ± 0.5	9.8 ± 1.3	2.3 ± 0.3	0/10	-	-	-