

## Arbuscular mycorrhizal fungi promote early flowering and prolong flowering in *Antirrhinum majus* L. by regulating endogenous hormone balance under field-planting conditions

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### Abstract

It is well documented that arbuscular mycorrhizal fungi (AMF) affect growth and nutrient absorption in host plants under pot conditions. However, their effects on reproductive growth in ornamental plants under field conditions are unknown. Our study evaluated the effects of AMF on flowering and physiological traits in snapdragon (*Antirrhinum majus*) under greenhouse field conditions. Seedlings were inoculated with *Funneliformis mosseae* (Nicolson & Gerd.) and without as controls. Results showed that AMF inoculation significantly increased plant height, stem diameter, phosphorus, and soluble protein; decreased soluble sugar; and had no effect on total nitrogen, carbon, and potassium. AMF colonization increased concentrations of abscisic acid (ABA), indol-3-acetic acid (IAA), gibberellin (GA<sub>3</sub>), and zeatin riboside (ZR); increased the ZR/IAA ratio; and reduced ABA/GA<sub>3</sub> and ABA/IAA+GA<sub>3</sub>+ZR ratios. AMF advanced flowering by five days and prolonged flowering by 13 days. Our study showed that AMF can promote flowering and prolong flowering in snapdragon, which may be due to the improvement of endogenous hormone equilibrium.

**Keywords:** arbuscular mycorrhizal fungi; flowering; endogenous hormone; snapdragon

### Introduction

Flowering phenology is an important ecological trait for plant populations (Liu *et al.*, 2018b; Song *et al.*, 2020). Although flowering phenology is generally genetically determined (Brachi *et al.*, 2010), it can be changed by many abiotic factors, such as temperature (Kopp *et al.*, 2020; Yang *et al.*, 2020), water resources (Song *et al.*, 2020; Vorkauf *et al.*, 2021), and soil fertility (Gondim *et al.*, 2020), as well as by biotic factors, such as herbivorous animals (Fogelström *et al.*, 2017; Ulrich *et al.*, 2020), and soil microorganisms (Lu *et al.*, 2018; Chaney and Baucom, 2020). Under controlled conditions, flowering time can be regulated by controlling temperature and applying water, fertilizer, plant growth regulators, and soil microorganisms (Liu *et al.*, 2018b; Lu *et al.*, 2018).

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Arbuscular mycorrhizal fungi (AMF) are soil microorganisms, which establish symbiotic relationships with most terrestrial plants (Genre *et al.*, 2020). AMF provide many benefits to host plants, such as enhancing plant growth, regulating hormone balance (Zhang *et al.*, 2019), and promoting absorption of mineral elements (Liu *et al.*, 2018b; Jiang *et al.*, 2021; Zhang and Feng, 2021). Zhang *et al.* (2019) found that AMF could regulate endogenous hormones in trifoliolate orange (*Poncirus trifoliata*) under drought stress, improve the ability of *Poncirus trifoliata* to absorb water and nutrients from the soil, and enhance drought tolerance in plants (Zhang *et al.*, 2019). He *et al.* (2017) observed that AMF inoculation increased the concentrations of gibberellin (GA) and zeatin riboside (ZR) and the ratios of GA/abscisic acid (ABA), ZR/ABA, and indoleacetic acid (IAA)+GA+ZR/ABA in peanut (*Arachis hypogaea*) and tomato (*Lycopersicon esculentum*), which promoted plant growth and increased insect resistance (He *et al.*, 2017). Liu *et al.* (2016) found that AMF enhanced IAA and ABA in leaves from trifoliolate orange (*Poncirus trifoliata*) growing in saline soils and, thus, increased its ability for osmotic regulation, leading to higher leaf water potential despite saline conditions (Liu *et al.*, 2016). Xie *et al.* (2018) showed that AMF could be used to regulate flowering in hyacinth (*Hyacinthus orientalis*), which led to earlier and prolonged flowering and could be due to changes in floral IAA and ZR levels that were caused by mycorrhization (Xie and Wu, 2018). However, at present, research on the effects of AMF to endogenous plant hormones is mainly focused on crops cultured in pots; whereas research on ornamental flowers is limited, especially under field conditions.

Snapdragon (*Antirrhinum majus*) is an important ornamental plant, which is commonly used for flower beds, flower borders, and cut flowers (Asrar *et al.*, 2012). The purpose of our study was to evaluate the effects of AMF on flowering and physiological traits of snapdragon.

## Materials and Methods

### *Study site*

The study was conducted in a greenhouse field of Laixi (36°86'N, 120°53'E), Qingdao, China from November 21, 2019 to April 10, 2020. The annual average temperature is 12.6 °C, the lowest in January with a monthly average temperature of -2 °C, and the highest in August with a monthly average temperature of 25.7 °C (Liu *et al.*, 2018a).

### *AMF, host plants and field soil preparation*

*Funneliformis mosseae* (T.H. Nicolson & Gerd.) was provided by the Institute of Mycorrhizal Biotechnology, Qingdao Agricultural University, and propagated with *Trifolium repens*, which was grown in pots under greenhouse conditions. Spores, mycorrhizal hyphae root segments in sand media were used as inocula.

Seeds of snapdragon (*Antirrhinum majus*, cultivar: 'General') provided by Beijing Forestry University Forest Science Company were surface-sterilized in 7% sodium hypochlorite solution for 10 min, then rinsed with sterilized distilled water for sowing.

The greenhouse field soil was sandy loam, which was deeply tilled and flattened, with a pH of 7.1, 1.07% organic matter, available N of 153 mg kg<sup>-1</sup>, available P of 94.8 mg kg<sup>-1</sup>, and available K of 150 mg kg<sup>-1</sup>.

### *Experimental design, sowing, inoculation and field management*

Two treatments were employed in this study: snapdragon seedlings inoculated with AMF and non-inoculated controls. The experiment was conducted with a randomized complete block design (RCBD) and three replicates. Plot area was 30 m<sup>2</sup> (3.0 m × 10.0 m), with row spacing of 0.3 m and 0.15 m spacing in rows.

Seeds were sown into a tray hole, filled with autoclaved, moist peat that was mixed with 300 *F. mosseae* spores, to culture the seedlings for transplanting. The same amount of autoclaved inocula and 5 mL of inoculum filtrate (50 µm filter) were added to tray holes as non-inoculated controls. On November 21, 2019,

seedlings with 3-4 leaves were transplanted in field soil according to the planting design above. When the seedlings were planted in the field, 5 g of inocula (approximately 200 AMF spores) were added to each hole for the inoculation treatment. The same amount of autoclaved inocula was added to each non-inoculated control.

Throughout the experiment, the greenhouse was well ventilated, and plants were managed regularly. Weeds and insect pests were controlled manually and with sticky boards, respectively.

#### *Assessments of plant growth, flowering, and mycorrhizal colonization*

Seedling height and stem diameter were measured every 20 d after field planting. Seedling height was the vertical height of the aerial part of the plant (Guo *et al.*, 2013; Liu *et al.*, 2018a). Thirty seedlings were measured for each treatment.

The duration of first-flowering (first flower open), full-flowering (>50% of flowers open), and final-flowering (>95% of flowers open) of racemes was estimated from the sowing day.

Roots were collected from plants 80 d after transplanting. A sample (15 g) of fresh fine roots was excised from each plot, washed, cleared, and stained with acid fuchsin. Percent of mycorrhizal colonization and arbuscule colonization was determined by the method of Biermann and Linderman (1981) with a BX50 Olympus microscope (Olympus Optical Co., Ltd., Tokyo, Japan) (Biermann and Linderman, 1981).

#### *Determination of physiological plant traits*

Mature leaf samples were collected every 20d after transplanting. Mature flowers were collected after 80 d from five plants. Samples were cleaned, weighed, immediately placed in liquid nitrogen, and stored in a -80 °C ultra-low-temperature freezer to determine the contents of soluble protein, GA<sub>3</sub>, ABA, IAA, and ZR. Remaining materials were dried in an 80 °C oven to constant weight for determining the contents of soluble sugar, total nitrogen, total carbon, phosphorus, and potassium. Soluble sugar was assayed by anthrone colorimetry (Li and Li, 2013). Soluble protein was assayed by Coomassie brilliant blue G-250 staining (Kaur and Kumar, 2020). Total nitrogen was assayed using a Kjeldahl apparatus (FOSS Kjeltac 8000, Denmark). Total carbon was assayed using a total organic carbon analyzer (X<sub>PERT</sub>-TOC/TN<sub>b</sub> m, Trace Elemental Instruments, Holland). Phosphorus and potassium were assayed by ICP-OES (Optima 8x00, PE, USA). Endogenous hormones were assayed by ultra-high pressure liquid chromatography-tandem mass spectrometry (Agilent Technologies, 1290/6460, USA).

#### *Statistical analyses*

Data were analysed by one-way analysis of variance (ANOVA) using SPSS Statistics 21.0 (IBM Corporation, Armonk, NY, USA). Duncan's multiple range test was used to compare treatments at a 5% significance level. Independent samples *t*-tests were used to analyse flower data between the two treatments. We also used SPSS Statistics for correlation analyses. All figures were generated with Origin 9.0 (OriginLab Co., Northampton, MA, USA).

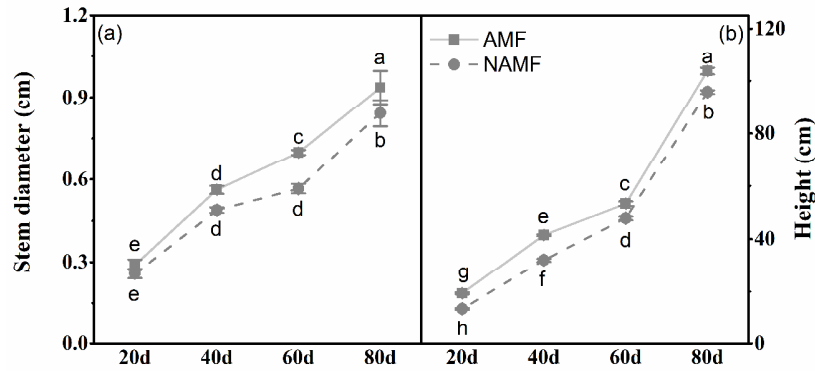
## **Results**

### *Mycorrhizal colonization*

Total mycorrhizal and arbuscule colonisations of snapdragon inoculated with *F. mosseae* were 79% and 46%, respectively. These colonisations were significantly higher than those of controls, which were 5% and little, respectively.

*Effects of AMF on growth and flowering of snapdragon*

The stem diameter of snapdragon inoculated with AMF was significantly greater than that of controls at 60 and 80 d after planting, while plant height was significantly higher than that of controls from 20 to 80 d (Figure 1).



**Figure 1** Stem diameter and height of snapdragon with and without AMF inoculation. AMF: arbuscular mycorrhizal fungi; NAMF: non-AMF control. Values are shown as mean  $\pm$  SE (n = 5). Different letters denote significant differences ( $P \leq 0.05$ ) from Duncan's test.

Compared to the controls, first-flowering and full-flowering days of snapdragon inoculated with AMF were significantly earlier, while final-flowering days were significantly delayed (Table 1). Flowering duration time was significantly longer in snapdragon inoculated with AMF than in controls (Table 1).

**Table 1.** Effects of AMF on flowering days in snapdragon during full-bloom period

Treatments	First-flowering days	Ful-flowering days	Final-flowering days	Flowering duration time
AMF	87.4 $\pm$ 0.5b	96.6 $\pm$ 1.1b	134 $\pm$ 1.6a	46.6 $\pm$ 1.2a
NAMF	92.8 $\pm$ 0.7a	104 $\pm$ 0.7a	126.4 $\pm$ 1.1b	33.6 $\pm$ 0.6b

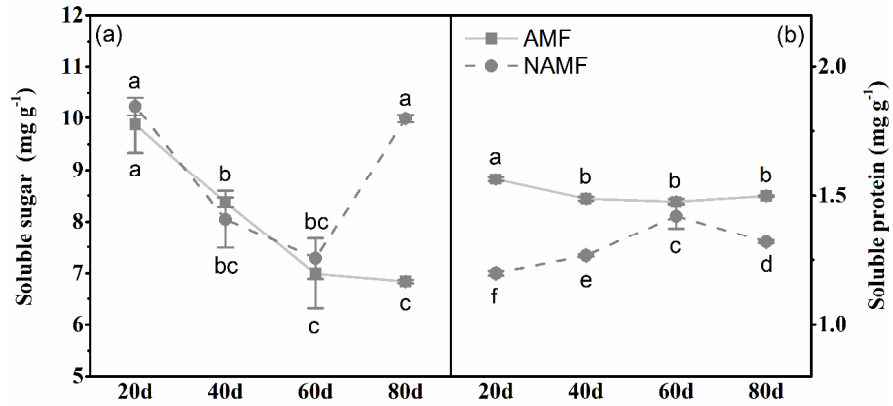
AMF: arbuscular mycorrhizal fungi; NAMF: non-AMF control. Different letters in the same row indicate significant differences between treatments from an independent samples t-test.

*Effects of AMF on soluble sugar and protein in snapdragon*

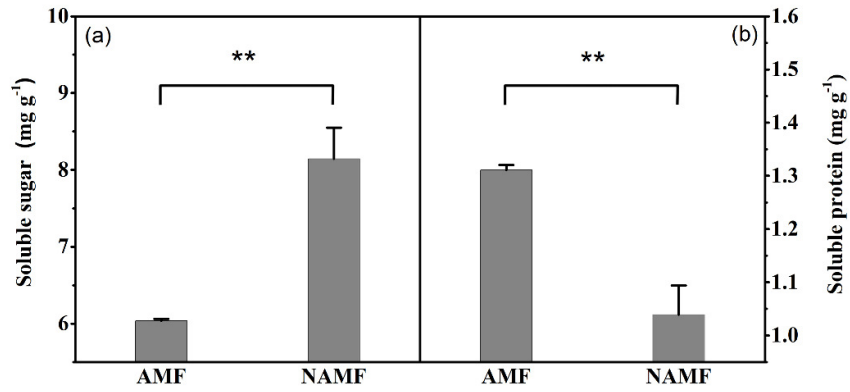
There was no significant difference in leaf soluble sugar between the two treatments from 20 to 60 d after planting, but soluble sugar was significantly lower in snapdragon inoculated with AMF than in controls at 80d (Figure 2a). Leaf soluble protein was significantly higher in snapdragon inoculated with AMF than in controls from 20 to 80d (Figure 2b). Flower soluble sugar was significantly lower in snapdragon inoculated with AMF than in controls (Figure 3a), while flower soluble protein was significantly higher in snapdragon inoculated with AMF than in controls (Figure 3b).

*Effects of AMF on minerals and carbon in snapdragon*

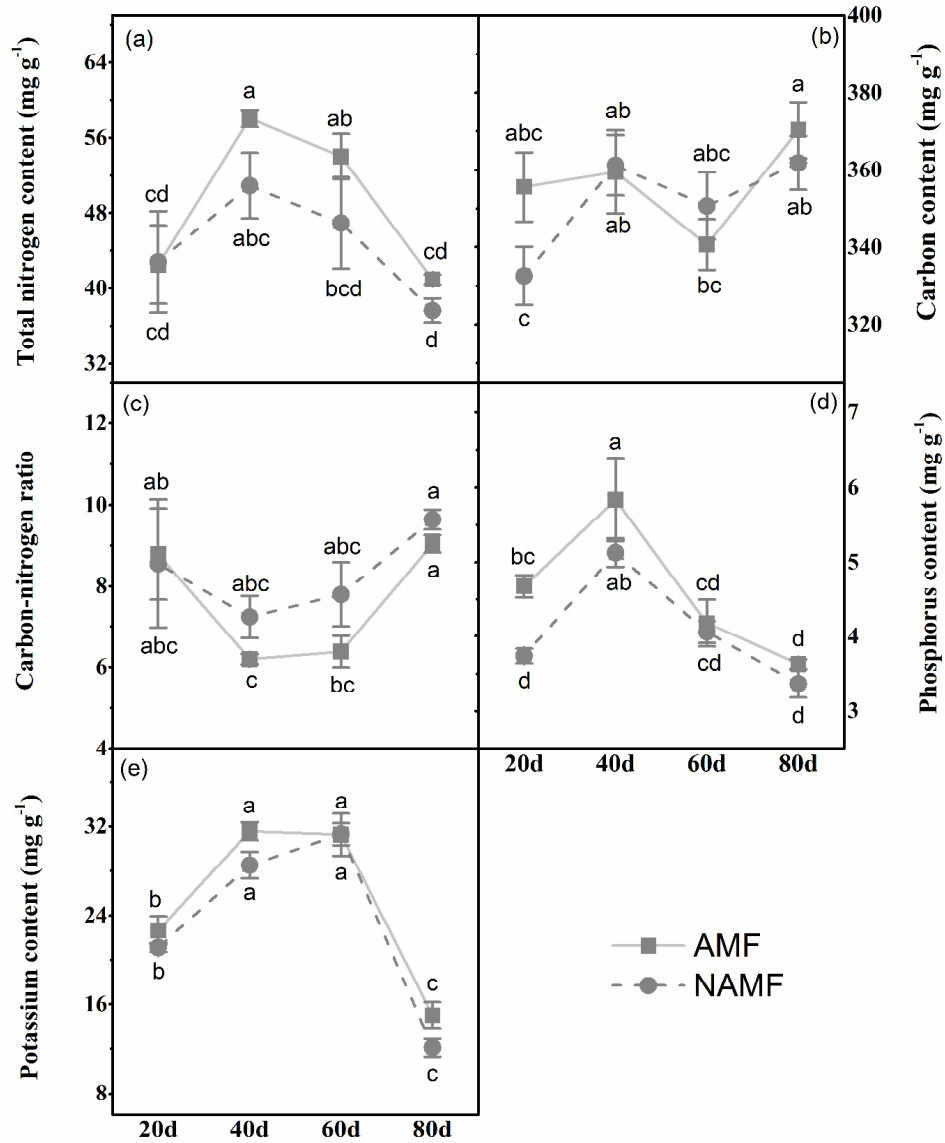
There were no significant differences in total nitrogen, phosphorus, carbon, and the carbon-nitrogen ratio in leaves between the two treatments (Figure 4). Total nitrogen and phosphorus increased and then decreased, reaching their highest values at 40 d (Figure 4a, d). Total carbon fluctuated: increasing, then decreasing, and finally increasing (Figure 4b). The carbon-nitrogen ratio decreased and then increased, reaching its maximum at 80 d (Figure 4c). Potassium increased and then decreased, reaching its highest value at 60 d (Figure 4e). Phosphorus in flowers was significantly higher in snapdragon inoculated with AMF than in controls (Figure 5d).



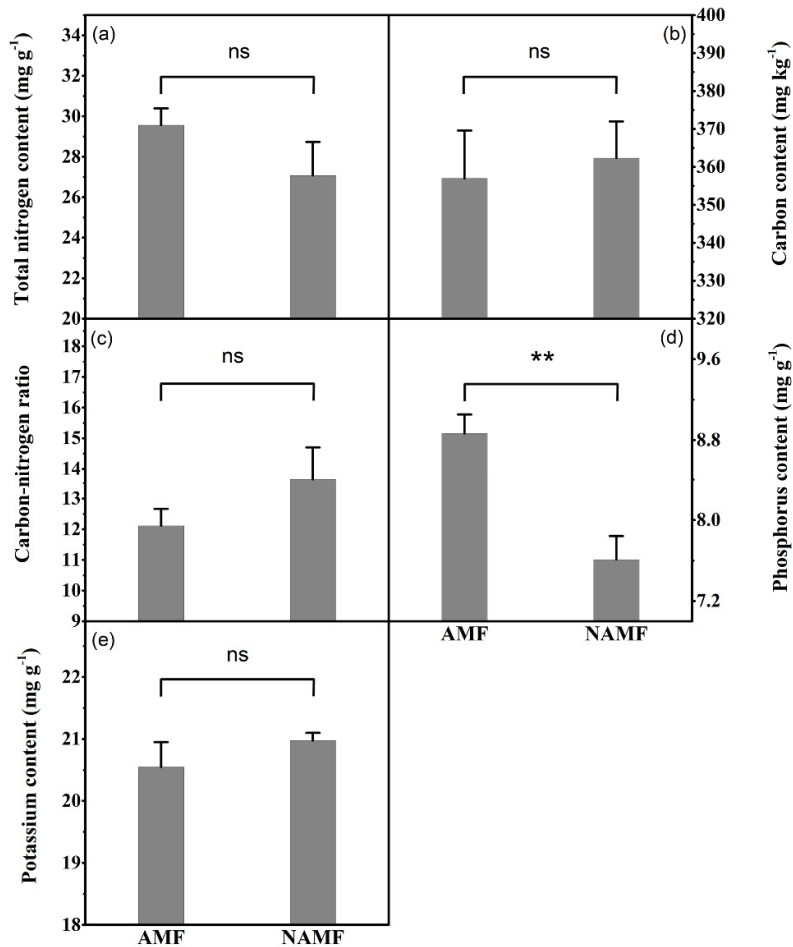
**Figure 2** Effects of AMF on soluble sugar and soluble protein in leaves of snapdragon  
 AMF: arbuscular mycorrhizal fungi; NAMF: non-AMF control. Values are shown as mean ± SE (n = 5). Different letters denote significant differences (P ≤ 0.05) from Duncan's test.



**Figure 3** Effects of AMF on soluble sugar and soluble protein in flowers of snapdragon  
 AMF: arbuscular mycorrhizal fungi; NAMF: non-AMF control. Values are shown as mean ± SE (n = 5). Asterisks indicate significant differences between treatments from an independent samples t-test: \*\*P ≤ 0.01 and \*P ≤ 0.05.



**Figure. 4** Effects of AMF on leaf stoichiometric traits in snapdragon  
 AMF: arbuscular mycorrhizal fungi; NAMF: non-AMF control. Values are shown as mean ± SE (n = 5). Different letters denote significant differences (P ≤ 0.05) from Duncan's test.



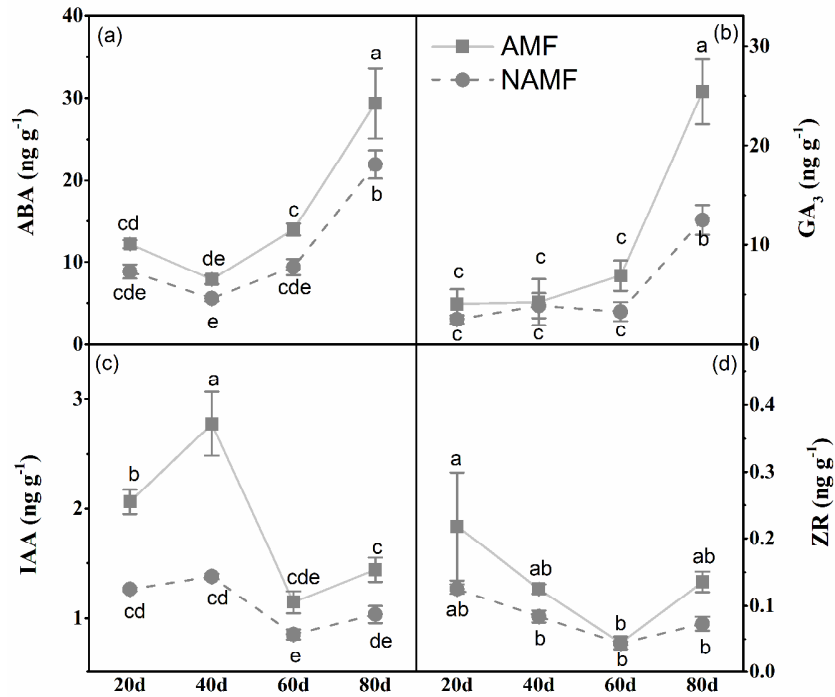
**Figure. 5** Effects of AMF on flower stoichiometric traits in snapdragon

AMF: arbuscular mycorrhizal fungi; NAMF: non-AMF control. Values are shown as mean  $\pm$  SE (n = 5). Asterisks indicate significant differences between treatments from an independent samples t-test: \*\*P  $\leq$  0.01, \*P  $\leq$  0.05, and ns P > 0.05.

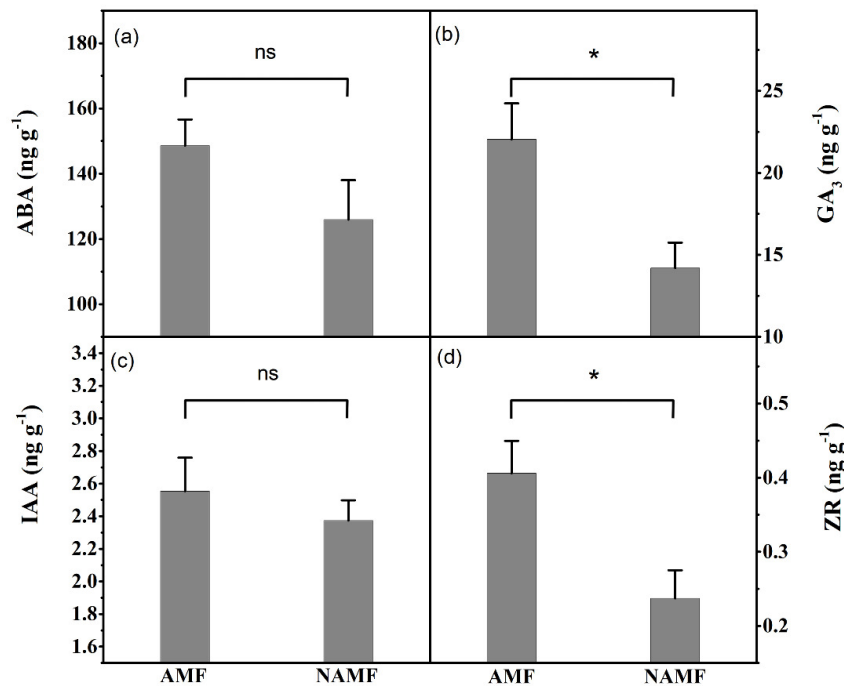
#### *Effects of AMF on endogenous hormones in snapdragon*

ABA and GA<sub>3</sub> concentrations in leaves were significantly higher in snapdragon inoculated with AMF than in controls at 80 d (Figure 6a-b). Leaf IAA was significantly higher in snapdragon inoculated with AMF than in controls from 20 to 40 d and at 80 d (Figure 6c). GA<sub>3</sub> and ZR concentrations in flowers were significantly higher in snapdragon inoculated with AMF than in controls (Figure 7b, d).

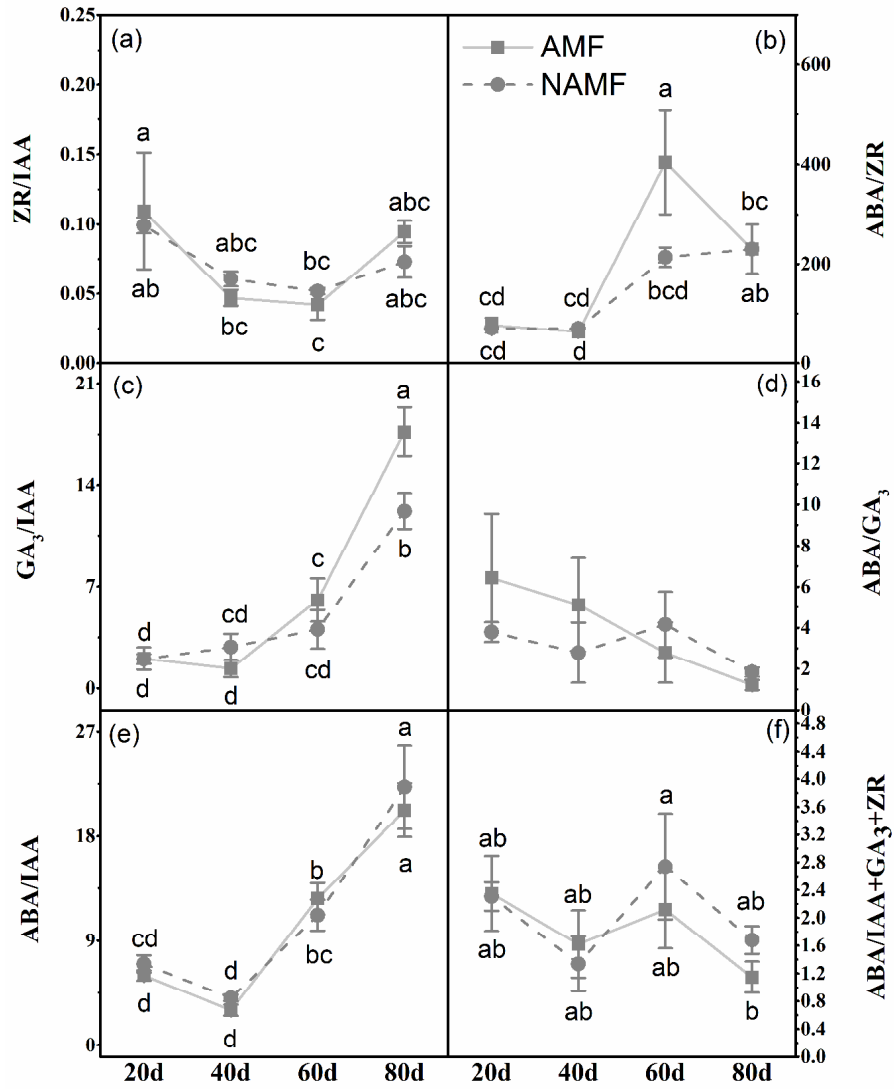
AMF significantly increased ABA/ZR and GA<sub>3</sub>/IAA ratios in snapdragon leaves at 60 and 80 d, respectively (Figure 8b-c). The ratio of ZR/IAA in flowers was significantly higher in snapdragon inoculated with AMF than in controls, while the ratios of ABA/GA<sub>3</sub> and ABA/IAA+GA<sub>3</sub>+ZR were significantly lower in snapdragon inoculated with AMF than in controls (Figure 9d, f).



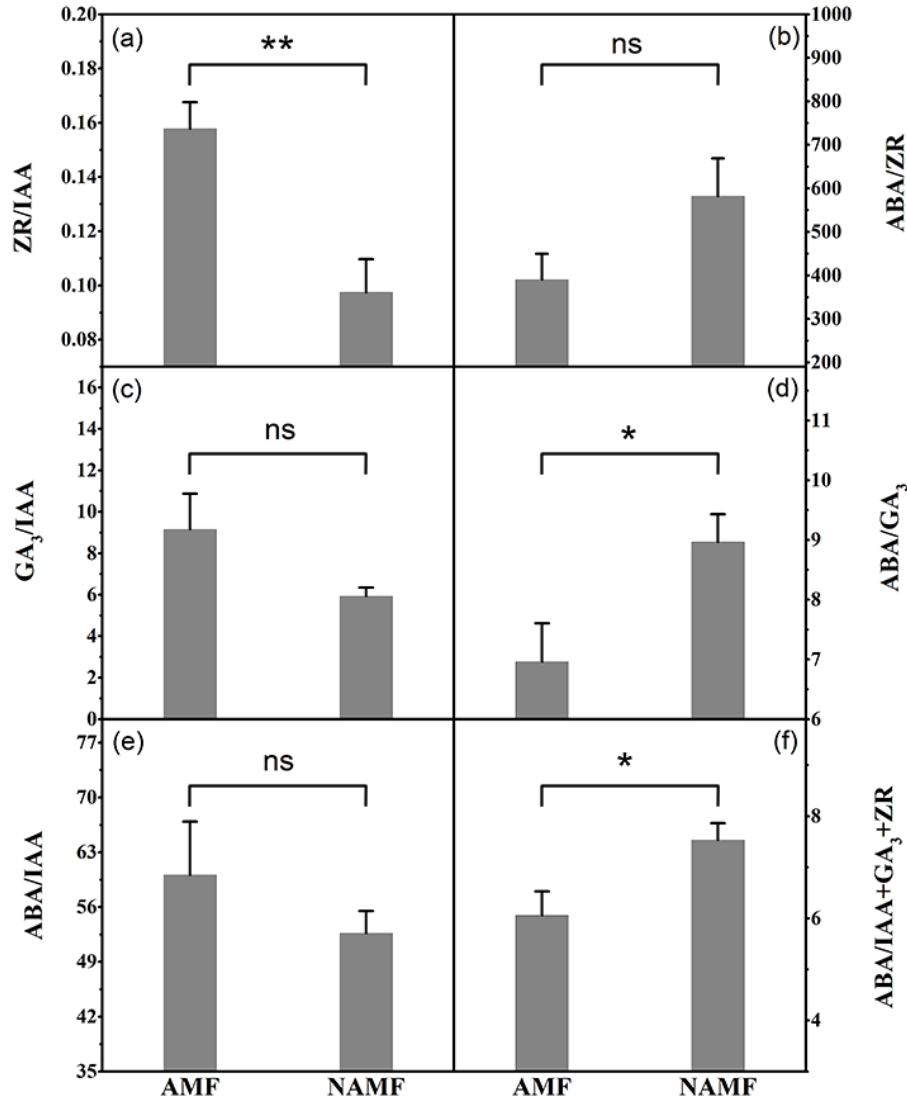
**Figure. 6** Effects of AMF on endogenous hormone levels in snapdragon leaves  
 AMF: arbuscular mycorrhizal fungi; NAMF: non-AMF control. Values are shown as mean ± SE (n = 5). Different letters denote significant differences (P ≤ 0.05) from Duncan's test.



**Figure. 7** Effects of AMF on endogenous hormone levels in snapdragon flowers  
 AMF: arbuscular mycorrhizal fungi; NAMF: non-AMF control. Values are shown as mean ± SE (n = 5). Asterisks indicate significant differences between treatments from an independent samples t-test: \*\*\*P ≤ 0.001, \*\*P ≤ 0.01, and \*P ≤ 0.05.



**Figure 8** Effects of AMF on endogenous hormone ratios in snapdragon leaves  
 AMF: arbuscular mycorrhizal fungi; NAMF: non-AMF control. Values are shown as mean ± SE (n = 5). Different letters denote significant differences (P ≤ 0.05) from Duncan's test.



**Figure. 9** Effects of AMF on endogenous hormone ratio in flowers of snapdragon  
 AMF: arbuscular mycorrhizal fungi; NAMF: non-AMF control. Values are shown as mean  $\pm$  SE (n =5). Asterisks indicate significant differences between treatments from an independent samples t-test: \*\*\*P  $\leq$  0.001, \*\*P  $\leq$  0.01, and \*P  $\leq$  0.05.

*Correlation analyses between endogenous hormones and flowering in snapdragon*

In leaves, GA<sub>3</sub>, ZR, and GA<sub>3</sub>/IAA were significantly negatively correlated with first-flowering and full-flowering times. Among these, the greatest correlation was between ZR and first-flowering time, and GA<sub>3</sub> had greater correlation with full-flowering time than first-flowering time. IAA was significantly positively correlated with final-flowering time and flowering duration time, while GA<sub>3</sub> was significantly positively correlated with flowering duration time. Overall, IAA had the greatest positive correlation with final-flowering time and flowering duration time (Table 2).

In flowers, GA<sub>3</sub> had a significant negative correlation with first-flowering time and a significant positive correlation with flowering duration time. ZR and ZR/IAA were significantly positively correlated with final-flowering time and flowering duration time. ABA/GA<sub>3</sub> and ABA/IAA+GA<sub>3</sub>+ZR were significantly positively correlated with first-flowering and full-flowering times. Overall, first- and full-flowering times had the greatest

correlation with ABA/IAA+GA<sub>3</sub>+ZR, whereas final-flowering time and flowering duration time had the greatest correlation with ZR/IAA (Table 3).

**Table 2.** Correlation analysis between endogenous hormones in leaves and flowering time in snapdragon

Hormone and ratio	First-flowering time	Full-flowering time	Final-flowering time	Flowering duration time
ABA	-0.523	-0.544	0.364	0.482
GA <sub>3</sub>	-0.703*	-0.704*	0.571	0.704*
IAA	-0.607	-0.570	0.706*	0.756*
ZR	-0.718*	-0.653*	0.561	0.704*
ABA/IAA	0.022	-0.020	-0.271	-0.199
ABA/GA <sub>3</sub>	0.416	0.415	-0.447	-0.493
ABA/ZR	0.443	0.350	-0.200	-0.332
ABA/IAA+GA <sub>3</sub> +ZR	0.397	0.392	-0.442	-0.481
GA <sub>3</sub> /IAA	-0.641*	-0.684*	0.362	0.532
ZR/IAA	-0.524	-0.467	0.199	0.367

\*\* Correlation is significant at the 0.01 level

\* Correlation is significant at the 0.05 level

**Table 3.** Correlation analysis between endogenous hormones in flowers and flowering time in snapdragon

Hormone and ratio	First-flowering time	Full-flowering time	Final-flowering time	Flowering duration time
ABA	-0.260	-0.217	0.625	0.550
GA <sub>3</sub>	-0.646*	-0.620	0.561	0.672*
IAA	-0.200	-0.238	0.228	0.246
ZR	-0.557	-0.576	0.653*	0.699*
ABA/IAA	-0.176	-0.110	0.464	0.400
ABA/GA <sub>3</sub>	0.760*	0.740*	-0.327	-0.559
ABA/ZR	0.350	0.395	-0.548	-0.535
ABA/IAA+GA <sub>3</sub> +ZR	0.777**	0.768**	-0.308	-0.553
GA <sub>3</sub> /IAA	-0.497	-0.462	0.405	0.499
ZR/IAA	-0.597	-0.606	0.770**	0.798**

\*\* Correlation is significant at the 0.01 level

\* Correlation is significant at the 0.05 level

## Discussion

Previous studies have shown that AMF can promote early flowering (Garmendia and Mangas, 2012; Bona *et al.*, 2015), postpone flowering (Dubsky *et al.*, 2002; Nowak, 2004), or have no effect on flowering (Gaur and Adholeya, 2005). AMF can also prolong (Jin *et al.*, 2015) or shorten flowering duration (Banla *et al.*, 2015). Our results showed that AMF significantly advanced flowering and prolonged its duration, which may be due to the improvement of physiological metabolism in snapdragon by AMF.

Soil phosphorus can affect flowering duration and flower number (Liu *et al.*, 2018b) because phosphorus promotes flower development and induces flowering (McArthur and Eaton, 1989; Wang *et al.*, 2011). Although AMF can enhance the uptake of phosphorus and other nutrients by host plants (Smith and Smith, 2012; Jiang *et al.*, 2021), and subsequently improve flowering, this may play a minor role in the present study.

Endogenous hormones are important factors in regulating plant reproduction and flowering (Shamshiri *et al.*, 2012; Xie and Wu, 2018). AMF colonization may directly or indirectly regulate hormone balance, thus affecting flower development and flowering (Pozo *et al.*, 2015). For example, GA<sub>3</sub> plays an important role in flower formation in ornamental plants (Zhang *et al.*, 2014). Previous studies have shown that AMF inoculation

increases the concentration of ZR in plants, thus promoting growth and development in plants (Liu *et al.*, 2016; He *et al.*, 2017; Xie and Wu, 2018). Our study found that AMF significantly increased GA<sub>3</sub> and ZR in leaves and flowers during the flowering period of snapdragon. Correlation analyses confirmed that GA<sub>3</sub> and ZR had a very significant negative correlation with first-flowering and full-flowering times and a positive correlation with final-flowering time and flowering duration time; higher concentrations of GA<sub>3</sub> and ZR were associated with earlier first-flowering and full-flowering times, later final-flowering times, and longer flowering duration time. Increases in GA<sub>3</sub> and ZR, induced by AMF, may be why AMF promotes flower development and early flowering (Kobayasi and Atsuta, 2010).

Previous studies have shown that auxin plays an important role in plant morphogenesis, and AMF colonization can increase IAA levels in plants (Alabadí *et al.*, 2009; Xie and Wu, 2018; Zhang *et al.*, 2019). Our study found that AMF inoculation significantly increased leaf IAA during the growth cycle of snapdragon (except for at 60 d). Our correlation analyses also verified that IAA in leaves was significantly positively correlated with final-flowering time and flowering duration time; higher concentration of IAA was associated with longer final-flowering time and flowering duration time. This implies that AMF may regulate flower development and flowering by accelerating IAA synthesis.

Previous studies have shown that ABA affects flower bud development in ornamental plants and can stimulate plant senescence (Koshita *et al.*, 1999; Ronen and Mayak, 1981). Our study showed that increases in ABA concentration at the flowering stage in snapdragon had little correlation with flowering phenology. We speculated that AMF may not be the main factor affecting flowering duration time in snapdragon. Our results also confirmed that AMF colonization may directly or indirectly regulate plant hormone balance, thus affecting flower development and flowering.

The effects of hormones on flower formation is not only represented by a single hormone but also by the balance of multiple hormones (Li *et al.*, 2019). Previous studies found that higher ratios of ZR/IAA, ABA/IAA, ABA/GA<sub>3</sub>, and ABA/IAA+GA<sub>3</sub>+ZR promote flower bud differentiation (Mo *et al.*, 2015; Wan *et al.*, 2018; Yan *et al.*, 2019). AMF also promote hormone balance in host plants (Nowak, 2004; Bona *et al.*, 2015). We found that AMF had strong effects on regulating hormone balance, especially in ZR/IAA, ABA/GA<sub>3</sub>, and ABA/IAA+GA<sub>3</sub>+ZR ratios in flowers. Correlation analyses showed that ZR/IAA had a very significant positive correlation with final-flowering time and flowering duration time, whereas ABA/GA<sub>3</sub> and ABA/IAA+GA<sub>3</sub>+ZR ratios had very significant positive correlations with first-flowering and full-flowering times. Therefore, AMF can promote early flowering and prolong flowering in snapdragon by adjusting endogenous hormone balance in snapdragon.

## Conclusions

AMF can improve plant growth and flowering in snapdragon by advancing flowering by five days and prolonging flowering by 13 days. Our results showed that AMF can regulate flowering by controlling endogenous hormone levels and balance in reproductive organs. Therefore, AMF can be employed to regulate early flowering and/or prolong flowering in the field. Future work should examine mycorrhizal effects on transcriptional levels of relevant flowering genes in snapdragon and the interaction between mycorrhizae and other elements (Ca, Mg, *et al.*) on flowering in snapdragon.

## Authors' Contributions

S.G. designed the experiment. L.X. and Y.Z. performed the experiment. L.X. participated in writing the manuscript. S.G., X.H., and WL participated in revising the manuscript.

All authors read and approved the final manuscript.

### **Ethical approval** (for researches involving animals or humans)

Not applicable.

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### **Conflict of Interests**

The authors declare that there are no conflicts of interest related to this article.

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