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Hydrogen Peroxide and Strigolactones Signaling Are Involved in Alleviation of Salt Stress Induced by Arbuscular Mycorrhizal Fungus in *Sesbania cannabina* Seedlings

Cun-Cui Kong[1,2] · Cheng-Gang Ren[2] · Run-Zhi Li[1] · Zhi-Hong Xie[2] · Ji-Ping Wang[1]

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Abstract The arbuscular mycorrhizal symbiosis can alleviate salt stress in plants by altering strigolactone levels in the host plant. The aim of this study was to investigate the mechanism by which strigolactones enhance salt stress tolerance in arbuscular mycorrhizal *Sesbania cannabina* seedlings. Strigolactone levels, as determined by means of germination bioassay, gradually increased with treatment time of NaCl applied. Inhibition of NADPH oxidase activity and chemical scavenging of H_2O_2 significantly reduced strigolactone-induced salt tolerance and decreased strigolactone levels. The H_2O_2-induced strigolactone accumulation was accompanied by increased tolerance to salt stress. These results strongly indicated that elevated H_2O_2 concentration resulting from enhanced NADPH oxidase activity regulated strigolactone-induced salt stress tolerance in arbuscular mycorrhizal *S. cannabina* seedlings.

Cun-Cui Kong, Cheng-Gang Ren have contributed equally to this study.

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✉ Zhi-Hong Xie
zhxie@yic.ac.cn

✉ Ji-Ping Wang
sxndwjp@163.com

1 Shanxi Agricultural University, Taigu 030801, China

2 Key Laboratory of Biology and Utilization of Biological Resources of Coastal Zone, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, China

Keywords Arbuscular mycorrhizal · Salt stress · *Sesbania cannabina* · Strigolactones · Hydrogen peroxide · NADPH oxidase

Introduction

Saline-alkali stress is a serious ecological problem that limits food production, and has caused severe harm to the environment and agricultural yields (Manivannan and others 2007). To survive such stress, plants have established beneficial associations with a number of microorganisms present in the rhizosphere that can alleviate the stress symptoms (Badri and others 2009). One of the most intensively studied and widespread mutualistic plant–microorganism associations is that established with arbuscular mycorrhizal (AM) fungi. About 80% of terrestrial plants, including most leguminous plants, are able to establish this type of symbiosis with fungi of the division *Glomeromycota* (Smith and Read 2008). *Sesbania cannabina*, recognized as a soil-improving legume, is used as green manure to increase the production of many crops. It is widely adaptable to different adverse climatic conditions, such as drought, waterlogging, and soil salinity. Therefore, *S. cannabina* plant-AM fungi symbiosis might represent a good strategy for increasing resistance to soil salinity (Ren and others 2016).

Establishment and functioning of AM symbiosis requires a fine-tuned coordination between the two partners, which is based on a finely regulated molecular dialogue (Andreo-Jiménez and others 2015). The molecular dialogue, the so-called pre-symbiotic stage, starts with the production and exudation into the rhizosphere of strigolactones by the host plant. Strigolactones are recognized by AM fungi by an uncharacterized receptor which stimulates hyphal growth and branching, thereby

increasing the probability of encountering a host root (Akiyama and others 2005). In addition to functioning as molecular cues in the plant–AM fungus interaction, strigolactones act as host detection signals in the rhizosphere that stimulate seed germination for root-parasitic plants of the Orobanchaceae, including *Striga, Orobanche*, and *Phelipanche* species (López-Ráez and others 2011). In accordance with their role as signaling molecules in the rhizosphere, strigolactones are mainly produced in the roots and have been detected in root extracts of both monocot and dicot plants (Xie and others 2010).

The significance of strigolactones during the initial stages of mycorrhizal colonization is widely recognized. Moreover, emerging evidence indicates that strigolactones may also play a role in subsequent steps of the symbiosis in response to environmental stresses, such as salt stress and drought stress (Aroca and others 2013; Ruiz-Lozano and others 2016). Since 2008, strigolactones have been classified as a novel class of hormones that control a number of processes in plants (Gomez-Roldan and others 2008). Strigolactones are biosynthetically derived from carotenoids (López-Ráez and others 2008) by sequential oxidative cleavage by two carotenoid cleavage dioxygenases—CCD7 and CCD8—belonging to the apocarotenoids (Walter and Strack 2011), as is abscisic acid (ABA). In addition, strigolactones and ABA all play critical roles in the regulation of salt stress responses and AM symbiosis establishment (Aroca and others 2013; Pozo and others 2015).

Hydrogen peroxide (H_2O_2), a ubiquitous reactive oxygen species, is involved in the regulation of multiple plant responses to salt stress (Cheeseman and others 2007). Although high concentrations of H_2O_2 cause cell death, low concentrations of H_2O_2 perform regulatory roles in plant stress responses. In addition, H_2O_2 functions as a second messenger in phytohormone signaling of plant stress responses (Xia and others 2009). It has also been proposed that H_2O_2 plays a critical role in induced tolerance by activating or inducing stress response-related factors, such as antioxidant enzymes (Gechev and others 2006). It is well known that ABA interacts with H_2O_2 signaling, which is dependent on NADPH oxidase activity in response to salt stress (Kwak and others 2003). However, currently few studies have investigated whether H_2O_2 signaling is also involved in strigolactone-mediated alleviation of salt stress.

In the present study, we inoculated *S. cannabina* seedlings with AM fungi and monitored the H_2O_2 content and strigolactone levels, to examine the relationship between H_2O_2 signaling pathways and AM fungus-induced increase in strigolactone production in response to salt stress.

Materials and Methods

Plant Materials and Treatments

Seeds of *S. cannabina* (Retz.) Pers. were obtained from the Shandong Academy of Agricultural Sciences, Shandong, China. Before sowing, the seeds were sterilized in 5% sodium hypochlorite for 5 min and rinsed several times with distilled water. The seeds were germinated at 28 °C in distilled water and sown in trays containing autoclaved zonolite at 1 week. Subsequently, individual seedlings were transferred to 1-L pots containing autoclaved zonolite inoculated with 10 g inoculum (approximately 116 spores). The original inoculum of the AM fungus *Funneliformis mosseae* (BGC NM03D) was propagated in pot culture on *Trifolium repens* for 8 weeks and included infected roots, hyphae, spores, and substrates.

After 1 week of inoculation, the seedlings were treated with NaCl solutions. Six salinity levels were applied: 0, 20, 40, 60, 80, and 100 mM NaCl. Treatments were completely randomized and replicated three times. Each NaCl solution was applied to the medium at the rate of 100 ml per week, with five applications in total. The growth conditions were as follows: a 12-h photoperiod, temperatures of 25/17 °C (day/night), and light intensity of 600 μmol m^{-2} s^{-1}. Subsequently, the seedlings were irrigated three times per week using half-strength Hoaglands nutrient solution to maintain approximately 80% field capacity as determined by weighing the pots. Three-week-old seedlings were used for all treatments.

Reagents used as specific scavengers or inhibitors, comprising 5.25 mKat L^{-1} catalase (CAT), 3 mmol L^{-1} diphenyleneiodonium (DPI) (Ren and Dai 2012), and 2 μM TIS108, the most potent and specific strigolactone biosynthesis inhibitor (Ito and others 2011), were purchased from Sigma-Aldrich (St Louis, MO, USA). Exogenous signaling molecules used were 10 mM H_2O_2 (Xia and others 2009) and 1 μM GR24 (a synthetic strigolactone analogue) (Cuyper and others 2014). All exogenous signaling molecules and inhibitors were filtered using 0.22-mm-diameter microporous membranes before use. A 100-μl volume of the exogenous signaling molecule or inhibitor solution was sprayed directly onto the plant leaves. An equal volume of distilled water was applied as the control treatment. Unless stated otherwise, inhibitors were applied 1 day before application of exogenous signaling molecules.

Plant Biomass and Photosynthetic Parameters

Seven-week-old seedlings were used for determination of plant biomass and photosynthetic parameters. The fresh biomass of the seedlings was determined by weighing immediately after harvesting. Photosystem II efficiency

(ΦPSII) and non-photochemical quenching of chlorophyll fluorescence (NPQ) were simultaneously measured using an open photosynthetic system (LI-6400XTR, Li-Cor, Lincoln, NE, USA) equipped with a leaf chamber fluorometer (6400–40, Li-Cor).

Measurement of Arbuscular Mycorrhizal Fungi Colonization

The percentage mycorrhizal colonization of the roots was calculated using the gridline intersection method (Giovannetti and Mosse 1980), after staining with trypan blue (Phillips and Hayman 1970).

Strigolactone Analysis by LC/MS–MS and Germination Bioassay

One day after application of exogenous signaling molecules, 0.5 g of roots was ground in a mortar with liquid nitrogen and then extracted with 1 ml ethyl acetate in a 3-ml glass tube. The tubes were vortexed and sonicated for 10 min in a Branson 3510 ultrasonic bath (Branson Ultrasonics, Danbury, CT, USA). The samples were centrifuged for 5 min at 4000×*g* in a MSE Mistral 2000×*g* centrifuge (Mistral Instruments, Leicester, UK). The organic phase was carefully transferred to 1-ml glass vials and stored at −20 °C until use in the germination bioassays.

Identification of strigolactones by liquid chromatography–tandem mass spectrometry (LC–MS/MS) was conducted as reported previously (Yoneyama and others 2008). Mass spectrometry was performed with a Quattro LC mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray source. Strigolactones identified in root extracts from *S. cannabina* seedlings are shown in supplementary information (Supplemental Fig. 1).

The germination bioassays with *Phelipanche ramose* seeds followed the method of Yoneyama and others (2008). The surface sterilized *P. ramose* seeds, approximately 20 each, were placed on 6-mm glass fiber disks (Whatman) and approximately 90 disks were incubated in a 9-cm sterile Petri dish lined with a sheet of filter paper and wetted with 6 ml of sterile Milli-Q water. Seeds require preconditioning for 12 days at 21 °C in the dark before the seeds become responsive to germination stimulants. Then, the conditioned seeds were transferred to a 5-cm sterile Petri dish prepared as follows. Aliquots (50 µl) of root extract were added to a 5-cm Petri dish lined with filter paper. The solvent was allowed to evaporate before the Petri dish carrying the conditioned seeds was placed on the filter paper and treated with sterile Milli-Q water (650 µl). The synthetic germination stimulant GR24 (10^{-6} M) and demineralised water were included as positive and negative controls in each bioassay. The Petri dishes were sealed, enclosed in polyethylene bags, and placed in the dark at 25 °C for 7 days. Then the germinated and ungerminated seeds were counted using a stereoscope. Seeds were considered germinated when the radicle protruded through the seed coat.

Measurement of H_2O_2 and NADPH Oxidase Activity

One day after application of exogenous signaling molecules, seedlings were harvested for determination of NADPH oxidase activity and H_2O_2 content. The concentration of H_2O_2 was determined by monitoring the absorbance of titanium peroxide at 415 nm following the method of Brennan and Frenkel (1977). One unit of H_2O_2 was defined as the chemiluminescence caused by the internal standard of 1 µM H_2O_2 g^{-1} fresh weight.

The NADPH-dependent O_2^--generating activity was examined using a superoxide dismutase (SOD)-inhibitable ferricytochrome *c* reduction assay. An aliquot of crude enzyme extract was added to a reaction mixture consisting of 50 mM HEPES-KOH (pH 7.8), 100 mM EDTA, 50 mM ferricytochrome *c* and 100 mM NADPH in the presence or absence of SOD (200 U ml^{-1}, from bovine erythrocytes; Sigma-Aldrich) and incubated at room temperature for 30 s. The activity was based on the difference between absorbance at 550 nm with or without SOD and the absorbance coefficient of 21.0 mM^{-1} cm^{-1}.

Statistical Analysis

All data were analyzed using Microsoft Excel (Redmond, WA, USA). The values were represented as the mean ± SD of three replicates for each treatment. One-way ANOVA was performed with SPSS Statistics 17.0 software (SPSS, Inc., Chicago, IL, USA). Duncan's multiple range test was used to compare pairs of means at the $\alpha = 0.05$ significance level.

Results

Strigolactones Enhance Salt Stress Tolerance in *S. cannabina* Seedlings

In the seedlings inoculated with AM fungi, root colonization steadily increased with seedling growth and differed significantly between sampling times (Table 1). To determine whether strigolactones enhance salt stress tolerance in AM *S. cannabina* seedlings, we generated five groups of seedlings that contained different levels of strigolactones by application of GR24, a bioactive strigolactone analogue, and TIS108, a specific inhibitor of strigolactone biosynthesis. The photosynthetic capacity and biomass of non-mycorrhizal seedlings declined sharply with increasing

Table 1 Root colonization by *Funneliformis mosseae* of *Sesbania cannabina* seedlings

Time	3 WAS	5 WAS	7 WAS
AMF colonization	8.6 ± 0.54c	26.3 ± 3.1b	45.8 ± 3.73a

Values are means ± standard error of triplicate samples

Data were separated using Duncan's multiple range test at $p < 0.05$

WAS weeks after sowing

salt concentration. AMF treatment clearly restored the biomass loss and photophysiological damage (Fig. 1). Concurrently, endogenous strigolactones levels(*m/z* 383, *m/z* 356, and *m/z* 317) increased with increasing salt concentration in AM *S. cannabina* seedlings (Table S1). Treatment with GR24 enhanced fresh weight and dry weight biomass and ΦPSII, and reduced NPQ, whereas TIS108 treatment reduced fresh weight and dry weight biomass and ΦPSII, and elevated NPQ compared with those of water-treated mycorrhizal seedlings (Fig. 1). In addition, treatment with GR24 also enhanced plant biomass and ΦPSII, and reduced NPQ without AM fungus inoculation. These results indicated that strigolactone accumulation induced by AM fungi inoculation enhanced salt stress tolerance in *S. cannabina* seedlings.

Interdependence of H_2O_2 and Strigolactone Levels in AM *S. cannabina* Seedlings

The H_2O_2 concentration of AM *S. cannabina* seedlings increased significantly in response to salt treatment compared with water treatment (Fig. 2a), indicating that salt stress may trigger H_2O_2 biosynthesis in the seedlings. Concurrently, the total amount of *P. ramose* germination, along with the endogenous strigolactone levels, increased significantly under salt stress in AM *S. cannabina* seedlings

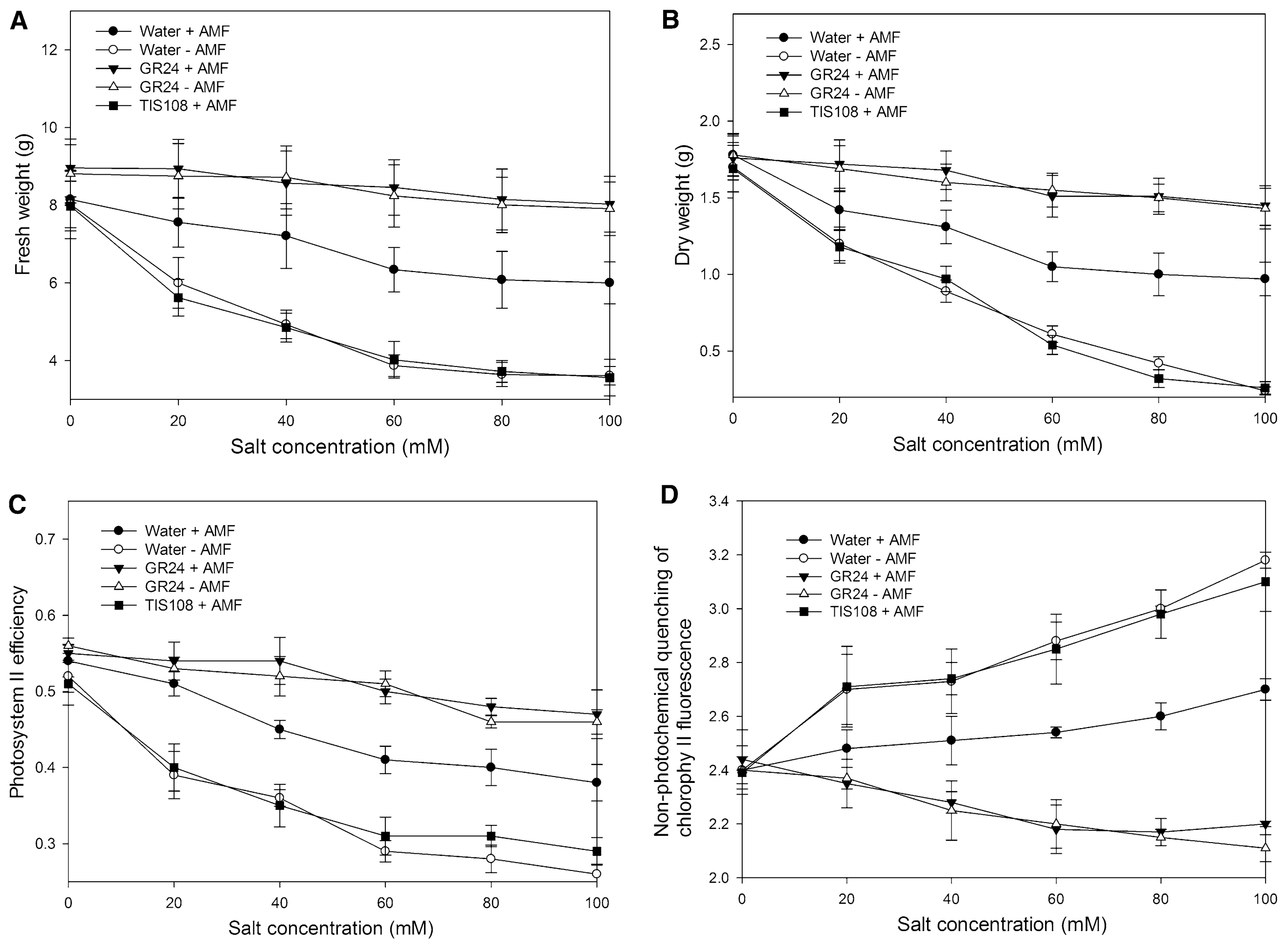

Fig. 1 Effect of strigolactone levels on plant biomass and photosynthetic parameters in *Sesbania cannabina* seedlings under salt stress 7 weeks after sowing. **a** Fresh-weight biomass, **b** dry-weight biomass, **c** photosystem II efficiency (ΦPSII), and **d** non-photochemical quenching of chlorophyll fluorescence (NPQ). Values are means of three independent experiments. The *error bar* represents the standard error

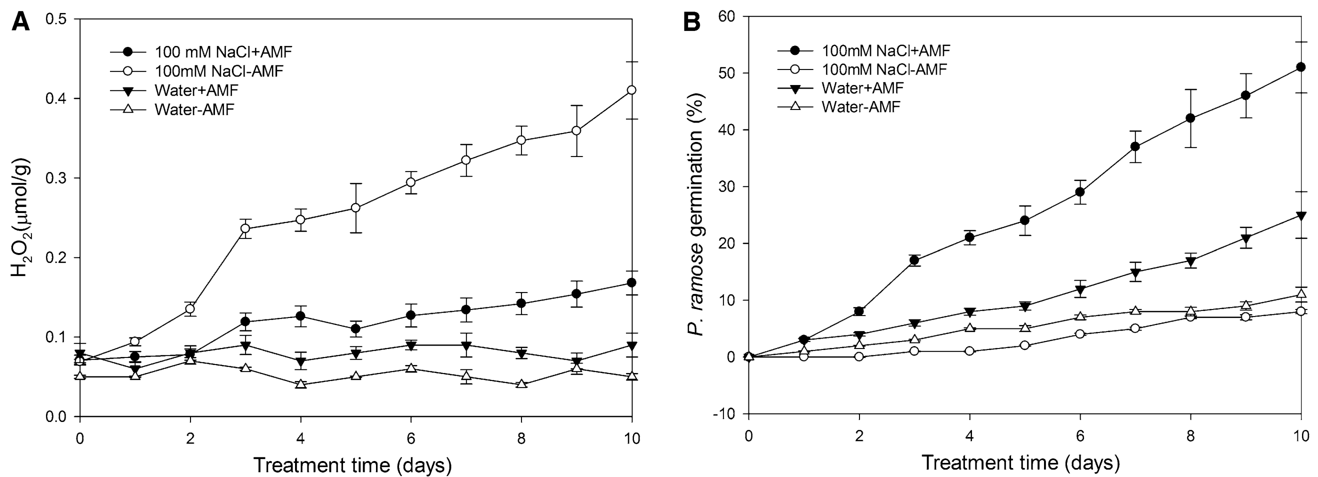

Fig. 2 Hydrogen peroxide (H_2O_2) concentration and accumulation of strigolactones (SLs) in *Sesbania cannabina* seedlings under different salt concentrations and AMF inoculation. **a** H_2O_2 production at 1-day intervals; **b** SLs production at 1-day intervals. Germination of *Phelipanche ramose* seeds induced by root extracts of *Sesbania cannabina* seedlings. Values are means of three independent experiments. The *error bar* represents the standard error

(Fig. 2b; Table S2). Interestingly, even without salt stress, the total amount of SL levels also gradually increased at a low concentration in AM *S. cannabina* seedlings (Fig. 2b; Table S2). In addition, H_2O_2 accumulation was abolished by DPI, a potent inhibitor of NADPH oxidase activity, and CAT, a H_2O_2 scavenger. To investigate whether H_2O_2 was involved in the AMF-induced strigolactone accumulation, DPI and CAT were applied. Both inhibitors suppressed not only H_2O_2 generation, but also the AMF-triggered strigolactone production (Fig. 3a, c; Table S3). The results suggested that H_2O_2 was important for AMF-induced strigolactone synthesis. When NADPH oxidase activity was inhibited by DPI treatment, strigolactone accumulation was reduced to control levels (Fig. 3b, c), which suggested that H_2O_2 production may be via the NADPH oxidase pathway. Taken together, these results suggested that H_2O_2 induced strigolactone accumulation in AM *S. cannabina* seedlings, which may be dependent on increased activity of NADPH oxidase.

Involvement of H_2O_2 in Strigolactone Levels Induced by AMF Alleviating Salt Stress

As shown in Fig. 3c, d, pre-treatment of CAT and DPI blocked SL levels and concurrently reduced dry weight in AM *S. cannabina* seedlings under salt stress. To determine whether H_2O_2 accumulation contributed to strigolactone-induced salt stress tolerance with AM fungus inoculation, we analyzed the effects of TIS108 on H_2O_2-induced tolerance of salt stress in AM *S. cannabina* seedlings. In GR24- or H_2O_2-treated seedlings, fresh weight and dry weight biomass and ΦPSII were greatly increased under salt stress in AM *S. cannabina* seedlings. Importantly, pretreatment with TIS108 completely abolished the protective effect of H_2O_2 on plant tolerance to salt stress in AM *S. cannabina* seedlings (Fig. 4a–c). Treatment with GR24 and H_2O_2 also alleviated significantly the increase in NPQ after NaCl treatment, and the protective effect of H_2O_2 was almost completely blocked by TIS108 application (Fig. 4d). These results strongly suggested H_2O_2-induced salt stress tolerance was depended on strigolactone accumulation in AM *S. cannabina* seedlings.

Discussion

A number of mechanisms responsible for increased resistance of host plants to salt stress following AM fungus inoculation has been intensively investigated (Liu and others 2015; De Almeida and others 2016). In this study, we proposed that enhanced strigolactone levels induced by an AM fungus may be responsible for the increased salt resistance in *S. cannabina* seedlings. The results showed AMF treatment significantly restored the biomass loss and photophysiological damage caused by salt stress (Fig. 1). Also, endogenous strigolactone levels (*m/z* 383, *m/z* 356, and *m/z* 317) accumulated gradually with increasing salt concentration (Table S1). Moreover, yjr strigolactone analogue GR24 enhanced and the strigolactone biosynthesis inhibitor TIS108 reduced sallt tolerance of mycorrhizal seedlings compared with water-treated mycorrhizal seedlings (Fig. 1). Several studies have reported that high levels of SL under stress conditions will lead to a corresponding increase in stress tolerance without AMF inoculation (Ha and others 2014; Bu and others 2014). Our results also showed that GR24 treatment alone could enhance salt tolerance of *S. cannabina* seedlings (Fig. 1). It strongly

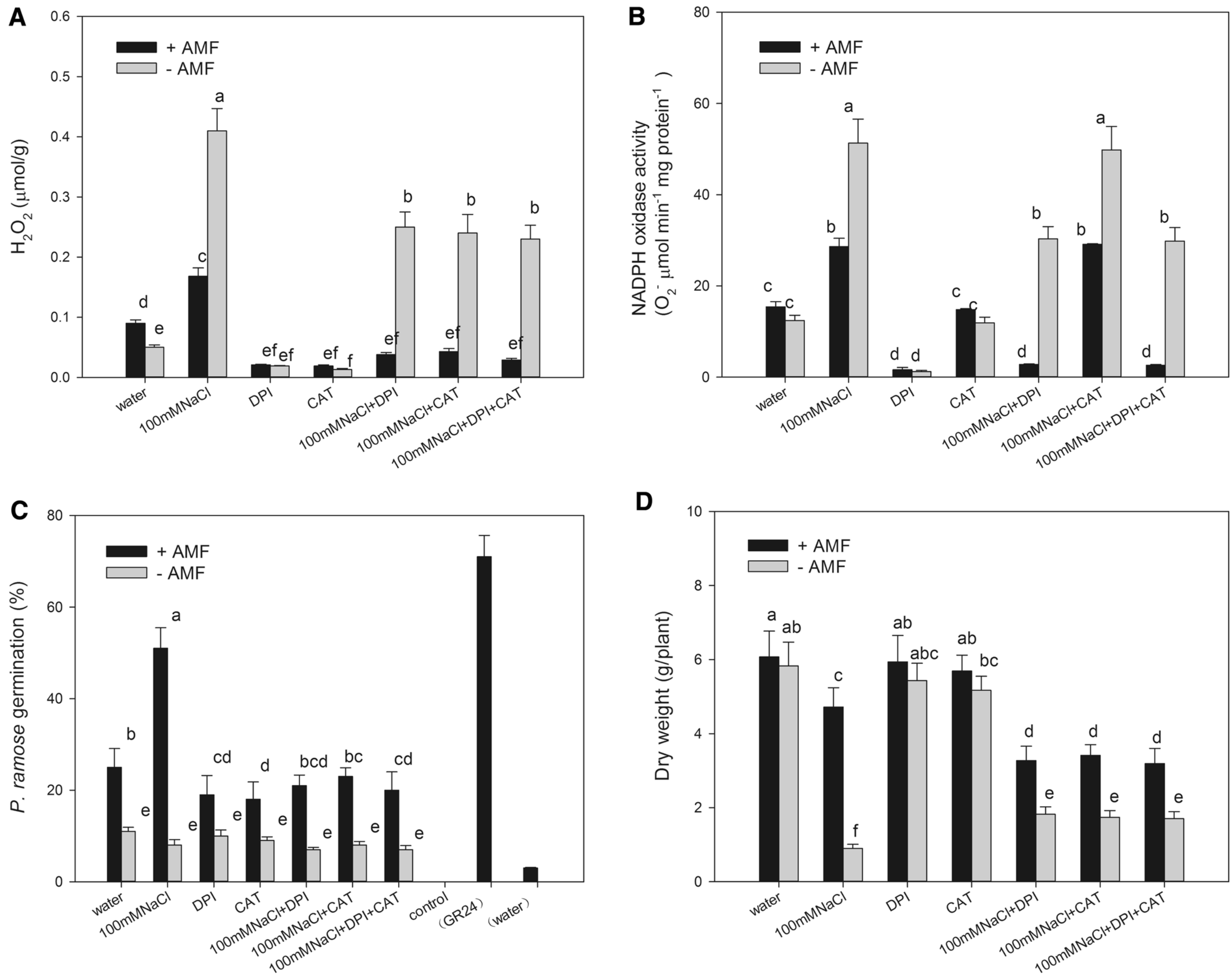

Fig. 3 Effect of hydrogen peroxide (H_2O_2) inhibitors on H_2O_2 concentration, NADPH oxidase activity and strigolactone (SLs) accumulation in arbuscular mycorrhizal *Sesbania cannabina* after treatment for 10 days. **a** H_2O_2 contents, **b** NADPH oxidase activities, and **c** SLs levels. Germination of *Phelipanche ramose* seeds induced by root extracts of *Sesbania cannabina* seedlings. **d** Dry weight (g). Treatments were inhibitors (3 mmol L^{-1} DPI or 5.25 mKat L^{-1} CAT) and 100 mM NaCl, which applied 1 day before arbuscular mycorrhizal fungal inoculation. Values are means of three independent experiments. The error bar represents the standard error. Data were separated using Duncan's multiple range test; different letters above the *error bars* indicate statistical significance at $p < 0.05$

suggested that GR24-induced salt stress tolerance is quantitative and is correlated with strigolactone levels in nature. Similarly, increased levels of strigolactones were detected in lettuce plants under salt stress in the presence of the AM fungus *Rhizophagus irregularis* (Aroca and others 2013). These observations in combination with our results indicate that strigolactones induced by an AM fungus may function as a phytohormone in plant tolerance against salt stress.

Strigolactones not only have diverse physiological functions, such as host-derived signals in the rhizosphere communication and phytohormone which participate in development and stress responses in plants, but also have multitudinous chemical structures. There are more than ten natural strigolactones isolated in various plants (Yoneyama and others 2009). All natural SLs isolated so far show a similar chemical structure, with a structural core consisting of a tricyclic lactone (the ABC-rings) connected via a characteristic enol ether bridge to a butenolide group (the D-ring) (Xie and others 2010). The D-ring is an important part of the molecule which is believed that SL activity resides in the CD junction (Ruyter-Spira and others 2013). In addition, it is known that each plant not only produces a single SL, but a blend of different SLs which depend on the species (Xie and others 2010; Ruyter-Spira and others 2013). Using LC/MS–MS, we identified four strigolactone candidates (*m/z* 383.34, *m/z* 337.61, *m/z* 355.91, and *m/z* 317.68) in *S. cannabina* root extracts that on fragmentation yield a daughter ion at *m/z* 97 (D ring), which is a

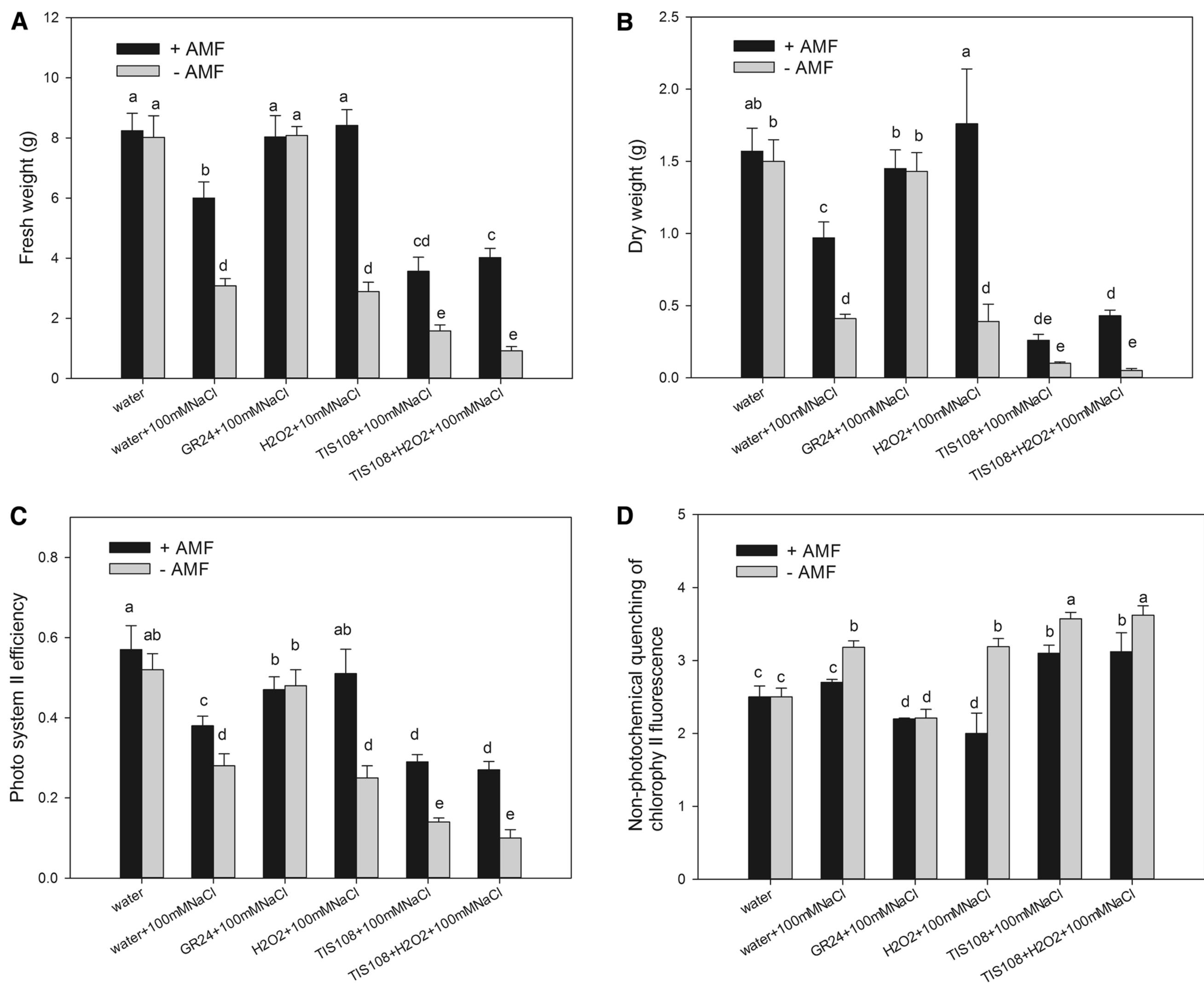

Fig. 4 Effect of H_2O_2 and SL inhibitor on plant biomass and photosynthetic parameters of arbuscular mycorrhizal *Sesbania canabina* seedlings under salt stress. **a** Fresh-weight biomass, **b** dry-weight biomass, **c** photosystem II efficiency (ΦPSII), and **d** non-photochemical quenching of chlorophyll fluorescence (NPQ). Inhibitors applied were 2 μM TIS108 or 10 mM H_2O_2. Values are means of three independent experiments. The *error bar* represents the standard error. Data were separated using Duncan's multiple range test; different letters above the *error bars* indicate statistical significance at $p < 0.05$

characteristic of strigolactones, by using the precursor ion mode (Gomez-Roldan and others 2008) (Fig. S1). The accumulation of three strigolactone candidates (*m*/*z* 383, *m*/*z* 356, and *m*/*z* 317) corresponded well with the results of *P. ramose* germination in Figs. 2b and 3c (Tables S2, S3).

Several authors have proposed that decreased H_2O_2 concentration is one mechanism by which AM fungi protect plants against salt stress (Hajiboland and others 2010; Garg and Bhandari 2012). In the present study, H_2O_2 levels were rapidly increased to a high concentration under salt stress without AMF colonization. Clearly, AMF treatment could delay the increase of H_2O_2 (Fig. 2a). A high concentration of H_2O_2 acts as an oxidative agent, whereas a low concentration may act as a signaling molecule (Xia and others 2009; Torres and Dangl 2005). Many studies also considered H_2O_2 as a signaling molecule in plant responses to diverse biotic and abiotic stresses (Xia and others 2009; Neill and others 2002). H_2O_2 accumulation was observed in *S. cannabina* seedlings in response to salt stress after AM fungal colonization, and gradually increased concomitant with plant growth at a low concentration (Fig. 2). Moreover, *P. ramose* germination, along with endogenous strigolactone levels (*m*/*z* 383, *m*/*z* 356, and *m*/*z* 317), was abolished by DPI, a potent inhibitor of NADPH oxidase activity, and CAT, a H_2O_2 scavenger (Fig. 3c; Table S3). These results suggested that the increase in H_2O_2 concentration induced by AM

establishment contributed to the induction of strigolactone accumulation under salt stress in *S. cannabina* seedlings.

In this study, we have provided several lines of evidence that H_2O_2 is involved in strigolactone-induced salt stress tolerance following AM fungus inoculation. DPI and CAT treatment blocked SL levels and concurrently reduced dry weight in AM *S. cannabina* seedlings under salt stress (Fig. 3d). Furthermore, the protective effect of H_2O_2 was almost completely blocked by TIS108 application in AM *S. cannabina* seedlings under salt stress indicating that H_2O_2-induced salt stress tolerance was dependent on strigolactone accumulation in AM *S. cannabina* seedlings, but not vice versa (Fig. 4d). The relationship between H_2O_2 and phytohormones under stress conditions has been studied extensively (Xia and others 2009, 2011). Many studies showed that ABA induces H_2O_2 accumulation in the apoplast, which is dependent on NADPH oxidase activity and plays an important role in ABA signaling (Kwak and others 2003). Additional studies have presented genetic and molecular evidence for the dynamic interplay between brassinosteroid- and ABA-induced H_2O_2 in tomato stress tolerance (Zhou and others 2014). It is likely that strigolactone-induced salt stress tolerance is mediated by a complex set of signal transcription pathways with H_2O_2 as a common signal molecule in the activation of the stress response.

In conclusion, we present strong evidence that H_2O_2 regulates the induction of strigolactone levels by an AM fungus during alleviation of salt stress in *S. cannabina* seedlings. Following perception of salt stress in AM plants, NADPH oxidase may be activated to produce H_2O_2. Further studies are needed to provide genetic evidence for the involvement of NADPH oxidase in H_2O_2-induced strigolactone generation and to identify the critical signaling components between strigolactone production and salt stress response in *S. cannabina* seedlings following AM fungus inoculation. Such studies will contribute to elucidation of the molecular mechanism of strigolactone-induced salt tolerance in AM plants.

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