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MECHANISM OF FLURIDONE-INDUCED SEED GERMINATION OF *CISTANCHE TUBULOSA*

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Abstract

Our previous study disclosed that fluridone, a synthesis inhibitor of abscisic acid (ABA), could stimulate seed germination in the holoparasitic plant *Cistanche tubulosa*. Nonetheless, the underlying mechanisms have not been thoroughly elucidated. In the present study, an attempt was made to reveal the mechanism of fluridone breaking seed dormancy in *C. tubulosa* and to determine the contribution of hormones in this process. The ABA level in seeds initially decreased following fluridone treatment and was subsequently maintained at a concentration of 31 $ng \cdot g^{-1}$ DW (dry weight) three days later. The contents of gibberellins (GAs) initially increased and subsequently were maintained at a level of 40 $ng \cdot g^{-1}$ DW after ten days. However, the increment of seed germination induced by fluridone was inhibited after the introduction of exogenous ABA or paclobutrazol (a synthesis inhibitor of GAs). Furthermore, inhibition from paclobutrazol was reversed by an additional treatment with exogenous GA3. When the ratio of endogenous GAs to ABA reached 4:3, *C. tubulosa* seeds initiated germination. By contrast, although the ratio of endogenous GAs to ABA content reached 2:1 by cold stratification, *C. tubulosa* seeds could not germinate unless exogenous GA3 was added. In summary, our current study revealed that (i) GAs and ABA play key roles for the seed germination of *C. tubulosa*, (ii) fluridone inhibited ABA biosynthesis but increased the concentration of GAs in seeds, and (iii) fluridone might initiate other processes associated with germination.

Key words: *Cistanche tubulosa*, Fluridone, Seed germination, Endogenous hormone, Parasitic plant.

Abbreviations: ABA, abscisic acid; ELISA, enzyme-linked immunosorbent assay; GAs, gibberellins; GA3, gibberellic acid

Introduction

Recently, fluridone has been reported to regulate seed conditioning and germination in many parasitic angiosperms. Fluridone can shorten the conditioning period required for the seeds of *Orobanche* spp. (Song *et al.*, 2005) and *Striga asiatica* (Kusumoto *et al.*, 2006) in response to a germination stimulant. It can also enhance the response of *Orobanche* spp. seeds to the artificial strigol-analogue germination stimulant GR24, even when the seeds are first conditioned at a suboptimal temperature and under water stress (Song *et al.*, 2006). In our previous investigations, we found that the percentage of *Cistanche tubulosa* seed that germinate can be significantly increased by fluridone treatment (Wang *et al.*, 2006). Fluoridone is known to act as an abscisic acid (ABA)-biosynthesis inhibitor, yet the underlying mechanisms of how fluridone effects seed conditioning and germination have not been fully disclosed. Chae *et al.* (2004) studied the effect of fluridone on seed conditioning and germination in *O. minor* and attributed the benefits to other perturbations rather than to the inhibition of ABA-biosynthesis. Moreover, a previous study found that fluridone could increase the germination percentage in *S. asiatica* seeds; however, the primary mechanism was not *via* inhibition of ABA biosynthesis (Kusumoto *et al.*, 2006). Therefore, the goals of this study were to confirm whether fluridone stimulates seed germination through inhibition of ABA-biosynthesis and to reveal other relevant mechanisms.

Cistanche tubulosa (Schenk) R. Wight, a member of *Orobancheace*, is a root holoparasitic plant mainly distributed in the deserts of the North and Northwest of China. It is also widely consumed as a precious medicinal herb in traditional Chinese medical practices for the treatment of kidney deficiency, female infertility, morbid leucorrhea, neurasthenia, and senile constipation induced by colonic inertia (Anon., 2010). However, because of its wide medicinal applications and consequent over-harvesting, it has been listed as one of the Class II plants needing protection in China. Even worse, the over-exploration of *C. tubulosa* aggravates the rate of desertification. Therefore, domestication of wild *C. tubulosa* could not only provide valuable tonic materials, but would also be beneficial in preventing desertification. Unfortunately, crucial seed dormancy was observed for *C. tubulosa* seeds. Seed germination cannot occur without exposure to natural germination stimulants released from the roots of a host plant, such as *Tamarix chinensis* (Li *et al.*, 1989; Chen *et al.*, 2011), thereby extensively hindering the cultivation of *C. tubulosa*.

Our previous study found that cold stratification breaks seed dormancy in *C. deserticola* through a mechanism that mainly relies on modifying the ABA contents in the seeds. Gibberellins (GAs) synthesis occurs widely during seed conditioning (Joel *et al.*, 1989, 1992, 1995; Takeuchi *et al.*, 1995; Zehhar *et al.*, 2002; Song *et al.*, 2005; Suzuki *et al.*, 1994), and an increment of GAs was observed in the seeds of *C. deserticola* after cold stratification, as expected (Chen *et al.*, 2009).

Therefore, to clarify the mechanisms involved in regulating seed germination in *C. tubulosa* by fluridone, particularly the roles of ABA and GAs, (i) the concentrations of endogenous ABA and GAs were measured during fluridone treatment and cold stratification, as well as germination time and percentage, and (ii) exogenous ABA, paclobutrazol (a synthesis inhibitor of GAs), and exogenous GA3 were screened to confirm further the effect of fluridone on endogenous ABA and GAs.

Materials and Methods

Plant material: *C. tubulosa* seeds were collected in 2012 from mature plants parasitizing on *T. ramosissima* at Yutian fields in Xinjiang, an autonomous region of China. Only seeds with a diameter greater than 0.5 mm were selected.

Chemicals: Fluridone, ABA, paclobutrazol, and GA3 were purchased from Sigma-Aldrich (Seelze, Germany). Stock solutions of fluridone, ABA, paclobutrazol, and GA3 were prepared with acetone, and the final concentration of acetone in the germination medium was not more than 0.1% (v/v).

Cold stratification of *C. tubulosa* seeds: Cold stratification of *C. tubulosa* seeds was performed by placing the seeds in moist sand with a water content of 4% (g/g) at 4°C, and the stratified seeds were subjected to the determination of GAs and ABA at different time points.

Germination of *C. tubulosa* seeds in different mediums: *C. tubulosa* seeds were surface-sterilized by immersion in 70% ethanol for 1 min followed by immersion 2% sodium hypochlorite containing 0.1% Tween-20 for 15 min (Zhou *et al*., 2004). Seeds were then rinsed with sterile water five times. After rinsing, 100 seeds were selected and placed on filter paper inside sterile 9 cm Petri dishes. Six types of incubation were performed for the *C. tubulosa* seeds as follows:

1. Incubation with water containing 0.1% (v/v) acetone: *C. tubulosa* seeds were incubated in 6 mL of sterile water (as a control).

2. Incubation with fluridone: *C. tubulosa* seeds were incubated in 6 mL of 10 $mg \cdot L^{-1}$ fluridone.

3. Incubation with fluridone and ABA: *C. tubulosa* seeds were incubated in 6 mL of 10 $mg \cdot L^{-1}$ fluridone containing different concentrations of ABA (0.01, 0.1, and 1 $mg \cdot L^{-1}$).

4. Incubation with fluridone and paclobutrazol: *C. tubulosa* seeds were incubated in 6 mL of 10 $mg \cdot L^{-1}$ fluridone containing different concentrations of paclobutrazol (0.03, 0.3 and 3 $mg \cdot L^{-1}$).

5. Incubation with fluridone and paclobutrazol, along with GA3: *C. tubulosa* seeds were incubated in 6 mL of 10 $mg \cdot L^{-1}$ fluridone containing 3 $mg \cdot L^{-1}$ paclobutrazol and 50 $mg \cdot L^{-1}$ GA3.

6. Incubation with GA3: *C. tubulosa* seeds treated with cold stratification at different time points were incubated with 6 mL of different concentrations of GA3 (0, 50, and 100 $mg.L^{-1}$).

The time course of seed germination was 40 d, and the time course of other germination assays was 24 d. The Petri dishes were sealed with Parafilm and wrapped in aluminum foil to provide absolute darkness. The Petri dishes were then placed in dark, controlled growth chambers at 25°C for germination. All experiments were replicated at least three times. The germination percentage was measured under a 20 × binocular dissecting microscope. Seeds were considered to have germinated when radicles emerged from the seed coat.

Measurement of GAs and ABA during fluridone treatment and stratification: Sampling was conducted 0, 3, 6, 10, 14, 18 or 24 d after 10 $mg \cdot L^{-1}$ fluridone treatment, or 0, 30, 60, 90, 120, or 150 d after cold stratification. The collected seeds (0.2 g) were quickly frozen in liquid nitrogen and stored at -80°C until use.

The extraction, purification, and measurement of endogenous levels of GAs and ABA by an indirect ELISA technique were performed as described in the literature (He, 1993) with some minor modifications. In brief, the seeds were homogenized in a mortar with liquid nitrogen and then extracted in cold 80% aqueous methanol fortified with butylated hydroxytoluene (1 mM) overnight at 4°C. Each supernatant was collected after centrifugation at 10000 g (4°C) for 20 min and filtered through a C18 Sep-Pak cartridge (Waters, Milford, MA). The effluent was then dried under N2-flow. The residue was dissolved using phosphate buffer solution (PBS, 0.01 M, pH 7.4) to determine the concentration of GAs and ABA. For ELISA-based quantitation, each well on a microtitration plate was coated with synthetic GAs or ABA ovalbumin conjugates in NaHCO3 buffer (50 mM, pH 9.6) and stored overnight at 37°C. Ovalbumin solution (10 mg·mL-1) was added into each well to block nonspecific binding. After incubating for 30 min at 37°C, standard GAs, ABA, samples and antibodies were added and the plates were incubated for a further 45 min at 37°C. The antibodies against GAs and ABA were obtained as described by Weiler *et al*. (1981). Next, horseradish peroxidase-labelled goat anti-rabbit immunoglobulin was added to each well and incubated for 1 h at 37°C. Finally, orthophenylenediamino substrate solution was added into each well and the enzyme reaction was conducted in the dark at 37°C for 15 min. The reaction was then terminated with 3 M H_2SO_4. The absorbance at 490 nm was recorded using a microplate reader. Calculations from the enzyme-immunoassay data were carried out following the protocols described in the literature (Weiler *et al*., 1981). In this study, the recovery percentage for each hormone was assayed by adding known amounts of standard hormone to a split extract. The recoveries were all above 90%, and all sample extract dilution curves paralleled the standard curves, indicating the absence of nonspecific inhibitors in the extracts.

Data analysis: The data shown in the figures are the means of three replicates with SEs. Fisher's least significant difference tests were performed for multiple comparisons of the treatment means at the 0.05 level of probability.

Results

Seed germination following treatment with fluridone: To determine the relationship between germination and endogenous ABA and GAs concentration levels, seed

germination percentages and contents of both ABA and GAs were recorded at different time points after fluridone treatment. Fig. 1 indicates that seed germination started at 12 days and was completed by 24 days, and the time courses for the change trends of ABA and GAs are shown in Fig. 2.

Changes in the endogenous ABA and GAs contents in seeds treated with fluridone: To affirm whether ABA biosynthesis was inhibited and whether GAs were involved in fluridone breaking *C. tubulosa* seed dormancy, the endogenous ABA and GAs contents in seeds were compared between water-incubation and fluridone-incubation.

The effect from sterilization was assessed first. The ABA level in dry, unsterilized seeds was approximately 218 ng·g^{-1} DW, whereas the level in sterilized seeds was 76 ng·g^{-1} DW (Fig. 2A). This indicated that sterilization reduced the level of ABA, which was mainly localized in the testa (Chae *et al.*, 2004). The GAs level in dry, unsterilized seeds was approximately 34 ng·g^{-1} DW, and the GAs level in sterilized seeds was 28 ng·g^{-1} DW (Fig. 2B). This showed that sterilization washed out smaller amounts of GAs that were mainly localized in the endosperm and embryo (Li *et al.*, 1989). Although sterilization could regulate the concentration levels of both ABA and GAs, it is necessary to protect seeds from pollution.

Incubation with water was also performed as a negative control. Following treatment with water, the ABA content increased rapidly with time to 148 ng·g^{-1} DW at 6 d. Conversely, the GAs content was not affected by water treatment at 3 days. After 3 d, however, it rapidly decreased and then stabilized at 6 d. It is uncertain why the GAs content reduced following water treatment, but presumably it was leached out.

In the first 3 days following fluridone treatment, the ABA content decreased sharply with time to 39 ng·g^{-1} DW, and subsequently decreased slowly with time. The mean content of ABA was approximately 31 ng·g^{-1} DW from 10 to 24 d. The GAs level was changed slightly by fluridone in the first 6 days. After 6 days, the content rapidly increased to 40 ng·g^{-1} DW at 10 d (Fig. 2B). The average GAs level was approximately 39 ng·g^{-1} DW from 10 to 24 d. The ratio of the GAs to ABA content was approximately 5:4 during this period.

Fluridone inhibited ABA biosynthesis when compared to control seeds treated with water. In addition, fluridone treatment increased the content of GAs in seeds during germination.

Inhibition of germination by ABA: To demonstrate further that the ABA content in seeds could inhibit germination, *C. tubulosa* seeds treated with a combination of fluridone and ABA and germination was assayed in the dark (Fig. 3). As expected, the introduction of exogenous ABA inhibited germination. This observation, together with the inhibition of seed germination by the high ABA level following treatment with water (Fig. 2A), suggested that increasing the ABA content inhibits seed germination.

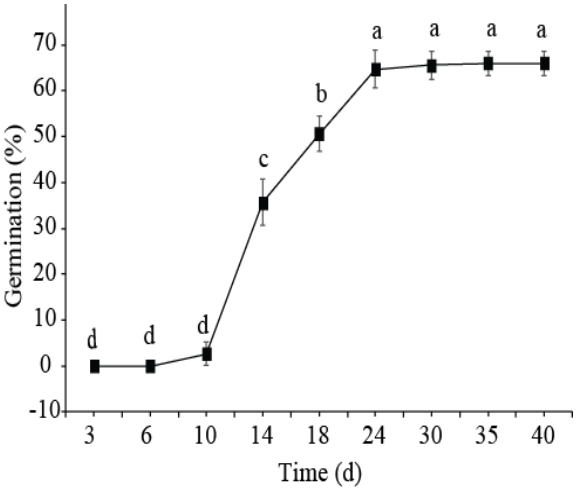

Fig. 1. Germination percentage of *C. tubulosa* seeds incubated in 10 mg·L^{-1} fluridone at different time points (3, 6, 10, 14, 18, 24, 30, 35, and 40 d). Data are the means of three replicates with SEs. Means designated with the same letter are not significantly different ($p<0.05$) according to Fisher's least significant difference test.

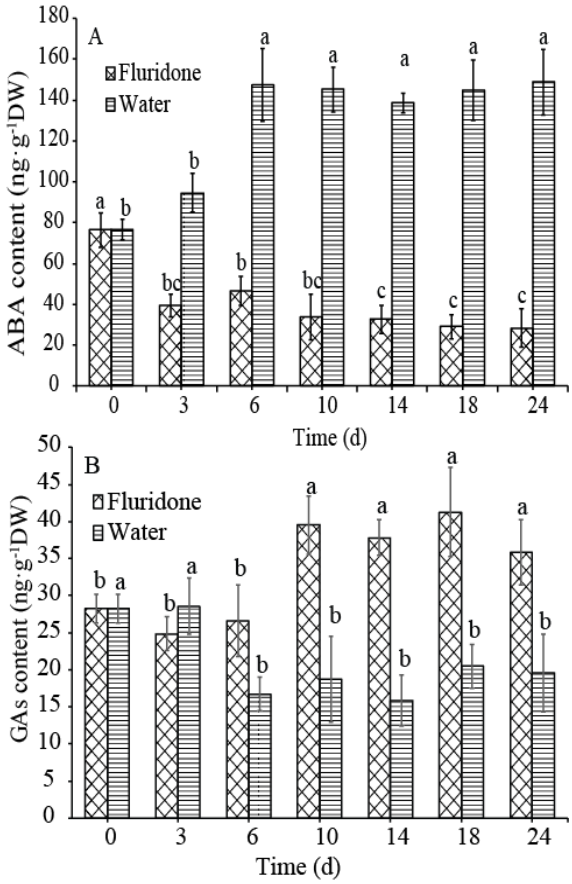

Fig. 2. Concentration-time chart of ABA (A) and GAs (B) following incubation of *Cistanche* seeds in 10 mg·L^{-1} fluridone or water at 0, 3, 6, 10, 14, 18, and 24 d. Data the means of three replicates with SEs. Means designated with the same letter are not significantly different ($p<0.05$) according to Fisher's least significant difference test.

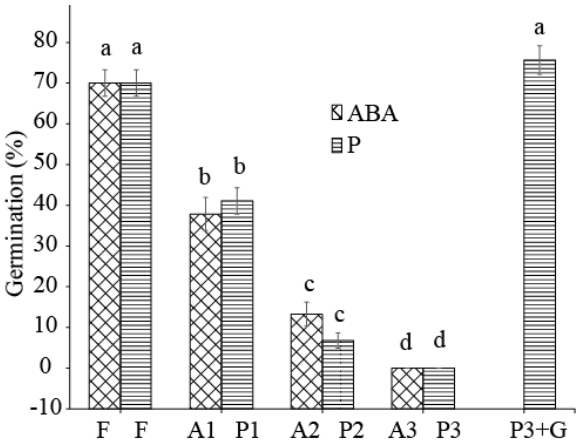

Fig. 3. Germination percentage of *Cistanche* seeds following incubation with 10 mg·L^{-1} fluridone (F), 10 mg·L^{-1} fluridone containing different concentration levels of ABA [0.01(A1), 0.1(A2) and 1(A3) mg·L^{-1}], 10 mg·L^{-1} fluridone containing different concentration levels of paclobutrazol [0.03 (P1), 0.3 (P2), and 3 (P3) mg·L^{-1}] or 10 mg·L^{-1} fluridone containing 3 mg·L^{-1} paclobutrazol and 50 mg·L^{-1} GA_3 (P3+GA). Data are the means of three replicates with SEs. For each germination percentage, means designated with the same letter are not significantly different (p<0.05) according to Fisher's least significant difference test.

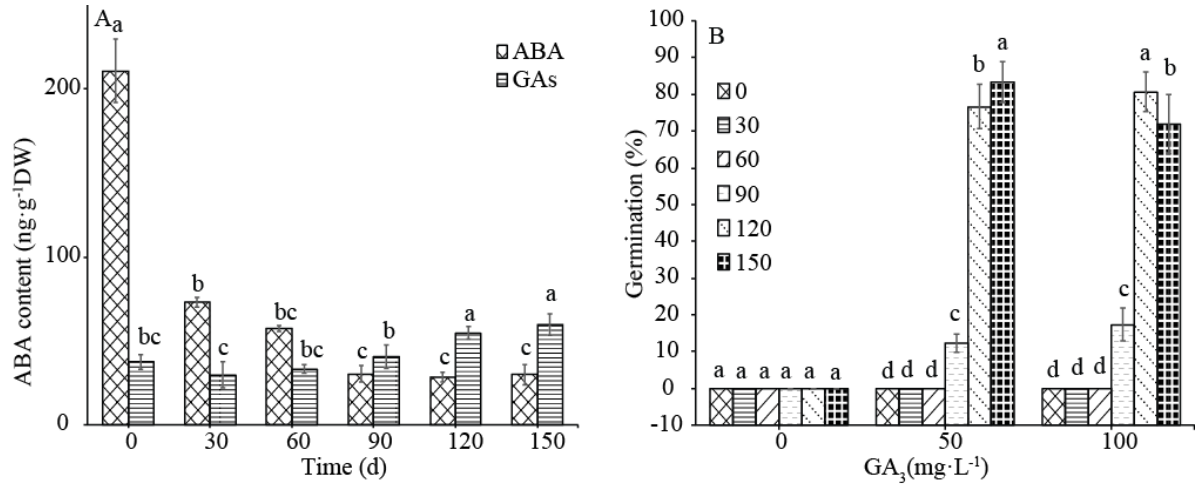

Fig. 4. Concentration-time chart of ABA and GAs following incubation of *Cistanche* seeds in cold stratification at 0, 30, 60, 90, 120 and 150 d (A), and germination percentage of *C. tubulosa* seeds incubated in 0, 50 and 100 mg·L^{-1} fluridone at different time points (0, 30, 60, 90, 120, and 150 d) (B). Data are the means of three replicates with SEs. For each ABA and GAs content and germination percentage, means designated with the same letter are not significantly different (p<0.05) according to Fisher's least significant difference test.

Inhibition of germination by paclobutrazol and its restoration by exogenous GA3: To determine whether endogenous GAs were synthesized during germination of *C. tubulosa* seeds treated with fluridone, the GAs biosynthesis inhibitor paclobutrazol was added to seeds together with fluridone. Inhibitors of GAs biosynthesis, particularly those inhibiting *ent*-kaurene oxidation, such as tetcyclacis, readily inhibit tomato and pepper seed germination. However, the inhibitory effect was reversed by GA_{4+7} (Khan 1994). The percentage of *C. tubulosa* seeds that germinated following treatment with fluridone in combination with paclobutrazol at different concentrations, or following treatment with fluridone, paclobutrazol, and exogenous GA3 was tested in the dark (Fig. 3). Germination decreased as the paclobutrazol concentration increased and was completely inhibited at 3 mg·L^{-1}. Moreover, the effect of paclobutrazol inhibition on seed germination was reversed by exogenous GA3. This trend, together with increased GAs level in seeds treated with fluridone (Fig. 2B), indicated that endogenous GAs synthesis occurred during seed germination.

Changes in the endogenous ABA and GAs contents in seeds treated with cold stratification: To further demonstrate that *C. tubulosa* seeds could germinate as long as the endogenous ABA and GAs contents reached a certain ratio and concentration, changes in the endogenous ABA and GAs contents were measured in seeds following cold stratification.

After dry seed was stratified for 30 days, the ABA level decreased rapidly from 211 ng·g^{-1} DW to 73 ng·g^{-1} DW. The ABA level reduced slowly from 30 to 90 d after cold stratification, and stabilized at approximately 29 ng·g^{-1} DW between 120 and 150 d after stratification (Fig. 4A). Overall, stratification treatment reduced the ABA content.

The GAs level increased gradually from 0 to 120 d after cold stratification. When the GAs level reached 40 ng·g^{-1} DW at 90 d (at which point the ratio of endogenous GAs to ABA levels reached 4:3), *C. tubulosa* seeds could not germinate (Figs. 4A and 4B). The GAs level stabilized at 57 ng·g^{-1} DW from 120 to 150 d (when the ratio of endogenous GAs to ABA levels reached 2:1); however, *C. tubulosa* seeds still could not germinate

unless exogenous GA3 was added (Figs. 4A and 4B). This result indicated that the contents of GAs in seeds treated with stratification increased; moreover, *C. tubulosa* seeds could not germinate even though the ratio of the endogenous GAs and ABA level reached 2:1. In addition, the endogenous ABA content (29 $ng \cdot g^{-1}$ DW) in seeds following stratification was less than that (31 $ng \cdot g^{-1}$ DW) in seeds following fluridone treatment.

Discussion

In the present study, fluridone, an inhibitor of ABA, effectively broke *C. tubulosa* seed dormancy. Because fluridone is an inhibitor of ABA synthesis, we presumed that fluridone was acting as an ABA biosynthesis inhibitor to break seed dormancy. This was confirmed by the direct measurement of ABA content during germination, which showed an accumulation of ABA in seeds treated with water, but a reduction of the ABA content in seeds treated with fluridone. Moreover, the breaking of dormancy by fluridone was counteracted when fluridone was combined with exogenous ABA treatment, indicating that fluridone inhibited ABA biosynthesis. Our previous results showed that after cold stratification decreased the ABA content of *C. deserticola* seeds, exogenous GA3 could break seed dormancy (Chen *et al*., 2009). Acetylcholine also broke *C. tubulosa* seed dormancy by reducing ABA content in the seeds (Yang *et al*., 2007). Fluridone could effectively shorten the conditioning time required for *O. minor* seeds to respond to strigol. However, the amount of ABA in the seeds or released into the conditioning media was not affected, i.e., fluridone did not inhibit ABA-biosynthesis (Chae *et al*., 2004). Fluridone and norflurazon could shorten the conditioning time required of *S. asiatica* seeds to the response of strigol. However, diflufenican did not exhibit promotive and protective effects on germination, even though it is also an ABA biosynthesis inhibitor (Kusumoto *et al*., 2006). A possible explanation for this difference may be that the mechanism of germination of *Cistanche* spp. differs from that of *Orobanch* spp. and *Striga* spp.

Paclobutrazol could counteract the effect of fluridone on breaking dormancy, and its counteraction could be reversed with GA3 treatment. Moreover, when the GAs in seeds treated with fluridone or water were compared over the time course of seed germination, the GAs content of seeds with fluridone increased over time while treatment with water reduced GAs content over time. These results suggest that GAs are involved in fluridone breaking seed dormancy in *C. tubulosa*. Our previous results also show that GAs are involved in cold stratification and exogenous GA3 breaking *C. deserticola* seed dormancy (Chen *et al*., 2009). It was suggested that GAs synthesis takes place during conditioning of *Orobanche* seeds because the application of paclobutrazol to the conditioning medium of *O. aegyptiaca* and *O. cumana* reduced the response to germination stimulation (Joel *et al.*, 1989, 1995). Recent studies have also shown that GAs synthesis occurs in some *Orobanche* spp. during conditioning (Joel, 2000; Zehhar *et al.*, 2002, Song *et al*., 2005).

Therefore, ABA biosynthesis was inhibited by fluridone and GAs were involved in fluridone breaking *C. tubulosa* seed dormancy. Comparing the content and ratio of endogenous GAs to ABA in seeds treated with fluridone, *C. tubulosa* seeds could germinate only when the average content of GAs and ABA stabilized at 40 and 32 $ng \cdot g^{-1}$ DW, respectively, and the ratio of GAs to ABA reached 4:3. The content of endogenous ABA of the *C. tubulosa* seeds treated with cold stratification (29 $ng \cdot g^{-1}$ DW) was less than with fluridone treatment (32 $ng \cdot g^{-1}$ DW). In addition, the content of endogenous GAs in seeds treated with cold stratification was equal or greater than with fluridone treatment. The ratio of endogenous GAs to ABA was equal or greater than 4:3; however, *C. tubulosa* seeds could not germinate unless exogenous GA3 was applied to the germination medium. These results suggest that fluridone induces various metabolic events associated with germination in *C. tubulosa* seeds.

Although fluridone treatment shortened the conditioning period required to respond to the germination stimulant strigol in *Striga* spp. and *Orobacnhe* spp. seeds, it could not release the seeds from dormancy (Song *et al.*, 2005; Kusumoto *et al.*, 2006; Chae *et al.*, 2004; Hisiao *et al.*, 1988). However, fluridone directly broke seed dormancy in *Cistanche* spp.. From the known mechanism of action of fluridone, there are similarities and differences among *Striga* spp., *Orobacnhe* spp. and *Citanche* spp. seed germination. The similarity is that fluridone initiates the formation or activation of receptor(s) for germination in these three-root parasitic plants (Song *et al*., 2005; Kusumoto *et al.*, 2006; Chae *et al.*, 2004). The difference, however, is that fluridone does not inhibit ABA biosynthesis in *Orobanch* spp. (Song *et al.*, 2005; Chae *et al.*, 2004), and it is still unclear whether fluridone inhibits ABA biosynthesis in *Striga* spp. (Kusumoto *et al*., 2006). In the present study, inhibition of ABA biosynthesis by fluridone treatment was demonstrated in *C. tubulosa*.

Conclusions

ABA biosynthesis was inhibited and GAs was involved in fluridone breaking *C. tubulosa* seed dormancy. In addition, fluridone initiated other processes associated with germination.

Low inoculation rate and long inoculation time (at least 8 weeks) create a bottleneck that limits the extension of *C. tubulosa* cultivation areas. Exploitation of our findings may lead to the development of *C. tubulosa* planting technology. If fluridone only regulates the hormone balance, as long as the germination conditions are suitable, *C. tubulosa* seeds treated with fluridone will germinate even in the absence of a host. The results of the current study demonstrate that fluridone is also involved in the formation or activation of receptor(s) for germination stimulants. This makes it possible to improve the inoculation rate and shorten the inoculation time. For example, endogenous hormones in *C. tubulosa* seeds could first be regulated by fluridone and seed priming technology. Then, a secondary dormancy could be induced by dehydration-storage technology. The use of dehydration-storage can effectively improve the inoculation

rate and shorten the inoculation time when planting *C. tubulosa*. Moreover, we should note that considerable work has been conducted to develop seed priming and dehydration-storage technology for *C. tubulosa* seeds. The results of this study have therefore laid a foundation to improve the inoculation rate and shorten the inoculation time of *C. tubulosa* seeds.

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