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Dormancy of *Nicotiana benthamiana* seeds can be broken by different compounds

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Abstract: The influence of after-ripening, sodium nitroprusside, potassium ferricyanide, cyanide, paclobutrazol and nitrite on germination of seeds of *Nicotiana benthamiana* was investigated as well as the influence of plant hormones such as gibberellins and abscisic acid. Dormancy of *N. benthamiana* seeds was broken by all treatments except treatments with abscisic acid, paclobutrazol and gibberellic acid (GA_3). Gibberellins had an interesting effect on dormancy breakage of studied seeds which was dependent on use of particular gibberellin: GA_3 or GA_{4+7}. Unlike GA_3, GA_{4+7} had broken seed dormancy.

Key words: abscisic acid; gibberellins; paclobutrazol; cyanide; sodium nitroprusside

Introduction

Seed dormancy is an important phenomenon in the life cycle of many plants but our understanding of it is still incomplete. It has been defined as a property of an individual seed that prevents germination when conditions would otherwise be suitable for germination (Korneef et al. 2002). It is common mainly among species of temperate regions for which is advantageous to delay germination until the season and conditions are favourable for seedling establishment (Bewley 1997).

Based on treatment with abscisic acid (ABA) and mutants with defects in ABA synthesis or signalling, ABA has been shown to play a crucial role in the acquisition and maintenance of seed dormancy (Korneef et al. 2002; Kucera et al. 2005).

Defined environmental cues can release dormancy in the imbibed seed state. Moist chilling (stratification) has been widely used as a pre-sowing treatment for breaking dormancy and enhancing the maximum rate of germination of dormant seeds of many different species (Valachovič 1991; Honěk & Martinková 1999; Fang et al. 2006) In many species dormancy release is also evident in the air-dry state during after-ripening (Moravcová & Frantík 2002; Leubner-Metzger 2005). Dry after-ripening, i.e. a period of dry storage for usually several months, is a frequently used method to relieve seed dormancy (Bewley 1997; Probert 2000; Kucera et al. 2005), and may represent a natural mechanism which can control dormancy in dry climates (Probert 2000).

Also a presence of light can be an important factor for seed germination because some species have photodormant seeds (Leubner-Metzger et al. 1995). Exposure to light often releases the final barrier to the completion of germination, thus terminating dormancy (Finch-Savage & Leubner-Metzger 2007). This phytochrome-mediated seed response to light can also be reversed in some cases by far-red light, until the seed is committed to the process of completing germination (Casal & Sánchez 1998).

Release of seed dormancy is usually associated with decrease of ABA level and an increase in gibberellin (GA) sensitivity or loss of GA requirement (Leubner-Metzger et al. 2005).

Gibberellins can counteract the effect of abscisic acid and can induce germination (Hilhorst & Karssen 1992; Leubner-Metzger et al. 2005) as well as they can substitute light in photodormancy and induce dark-germination of photodormant seeds (Leubner-Metzger et al. 1995). In addition to gibberellins other plant hormones (for example brassinosteroids and ethylene) are known to promote germination. However, unlike gibberellins brassinosteroids and ethylene cannot break photodormancy (Leubner-Metzger 2001). However, seed germination is not observed in a presence of inhibitors of ent-kaurene oxidase – uniconazole or paclobutrazol (Nambara et al. 1991; Li et al. 2005) due to inhibition of GA synthesis. The enzyme ent-kaurene oxidase catalyzes the three step oxidation of ent-kaurene to ent-kaurenoic acid in the GA biosynthesis pathway and when paclobutrazol is applied it binds to an iron atom in the ent-kaurene oxidase and so blocks GA synthesis (Swan et al. 2005).

Lots of very different compounds are known to

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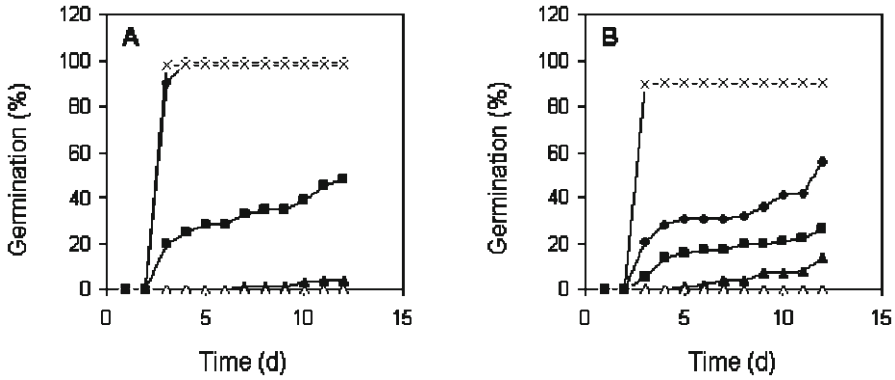

Fig. 1. After-ripening of *N. benthamiana* seeds under two different storage conditions: Time course of germination of (A) room temperature stored seeds and (B) refrigerator (4 °C) stored seeds: △ immediately after the harvest, ▲ after 2 weeks, ■ after 4 weeks, ◆ after 8 weeks × after 12 weeks.

break dormancy. They can be natural such as cyanide, smoke volatiles, hydrogen peroxide, nitrate and nitrite or synthetic such as azide (Roberts 1972; Hendricks & Taylorson 1974; Hilhorst & Karssen 1992; Debeaujon et al. 2000; Narimanov 2000; Flematti et al. 2004). Recently has been shown that sodium nitropruside and ferricyanide are also able to break dormancy. It was proposed that in both cases it is due to cyanide moiety (Bethke et al. 2006).

N. benthamiana has a short lifecycle and is easy to grow. Also it can be consider as a "small tobacco" in comparison with *Nicotiana tabaccum*, so it is easy to manipulate with.

Nicotiana benthamiana is highly interesting for germination studies as dormancy of its seeds is likely to be established at higher temperatures due to origin of this species. It is a native species of Australia (Horton 1981). Furthermore the species can be used for transformation (Kamachi et al. 2007).

Our work was aimed on examination of the influence of phytohormones (ABA, GA), nitrogen and cyanide containing compounds as well as on some environmental factors in dormancy release of *Nicotiana benthamiana* seeds.

Material and methods

Plant material

Seeds of *N. benthamiana* were obtained from plants grown in a greenhouse during summer months of June and July. Plants were grown under natural light and a temperature was changing according to weather. The temperature ranged from 20 °C to 35 °C. Seeds were harvested after a pod opened, dried for 24 hrs at room temperature, and than stored in tubes at −80 °C. For after-ripening, seeds were stored in paper bags at room temperature. For cold treatment, dry seeds were placed into tightly closed tubes and stored at 4 °C.

Chemicals

Chemicals were of analytical grade. Sodium nitroprusside (SNP), cyanide (KCN) and ± cis, trans-abscisic acid were purchased from Sigma-Aldrich, Germany. Potassium nitrate was purchased from Penta Chrudim, the Czech Republic, potassium nitrite from Lachema Brno, the Czech Republic, GA_3 (G 0907) and GA_{4+7} (G 0938) and paclobutrazol (P 0922) from Duchefa Biochemie, the Netherlands, ferricyanide (PFC) from ICN Biomedicals Inc., the USA.

Germination tests

Seeds of *N. benthamiana* were imbibed in 11-cm glass Petri dishes containing double layer of sterile filter paper (Whatman International Ltd, England) moistened with 10 mL of sterile distilled water containing desired compounds. The imbibition lasted three days except treatments with ABA when seeds were constantly in ABA solution. After imbibition seeds were washed with water and put into new Petri dish as described above containing pure water if not mentioned otherwise.

Seeds were incubated at 23 °C under cool white light 51μM (PAR) m^{-2} s^{-1} (Osram L18/20). The photoperiod was 16 hrs. For dark incubation Petri dishes were wrapped into aluminum foil. Germination tests were performed thrice using triplicate samples (each sample containing 50 seeds). Seeds were scored as germinated when radicle occurred. Figures show representative data out of 3 replicates.

Results

Seeds of *N. benthamiana* were released from seed dormancy by after-ripening at the room temperature (Fig. 1A) and also at 4 °C (Fig. 1B). After-ripening at room temperature was proved to be more efficient than cold treatment. The room temperature treated seeds were fully released from dormancy after eight weeks. It was four weeks earlier than in case of seeds treated with cold at 4 °C (Fig. 1).

Cyanide, ferricyanide and sodium nitroprusside break seed dormancy of *N. benthamiana* in dose-dependent manner as shown in Fig. 2. The higher concentrations of these compounds (100 μM and 250 μM) were more efficient in releasing dormancy. However, the use of 10 μM had an advantage that seeds were not affected by the toxic effect of these compounds. The mentioned seeds did not have to be washed to start germinate. The toxic effect was also observed on non-dormant seeds. The seeds were not able to germinate in the presence of 100 μM and 250 μM KCN, SNP and

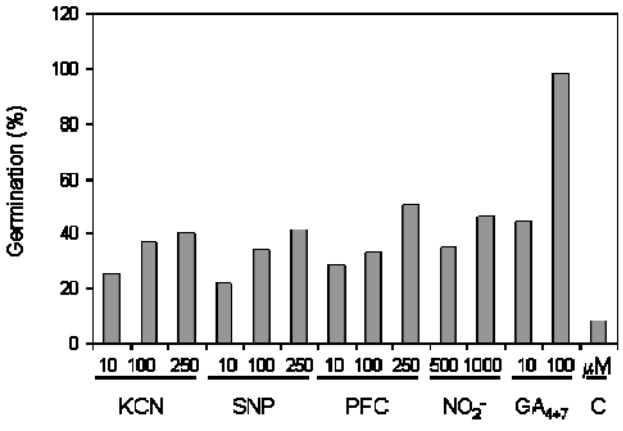

Fig. 2. Compounds breaking seed dormancy of *N. benthamiana* seeds. Germination scored 12 days after the end of imbibition.

PFC. Nevertheless they readily germinated after washing (data not shown).

Also nitrite promotes germination of *N. benthamiana* seeds but in much higher concentrations (500 µM and 1000 µM) than KCN, PFC and SNP (Fig. 2). However, seeds have not to be washed because nitrite has no toxic effect on germination (data not shown). Nitrate applied in same concentrations as nitrite was not capable of dormancy breakage (data not shown).

Exogenous application of GA_3 on dormant and non-dormant seeds had no effect on their germination (Fig. 3A). However, GA_{4+7} promoted germination of dormant seeds in dose-response manner and had no effect on germination of non-dormant seeds, as shown in Figs 2, 3B. GA_{4+7} also overcome the inhibition of germination by darkness (data not shown).

Figure 3C shows how paclobutrazol inhibited germination of non-dormant seeds of *N. benthamiana*. The response of non-dormant seeds was again dose-dependent where 10 µM were not effective in inhibition of germination but 100 µM reduced germination from 98 % of control to 27% and 200 µM resulted in no germination.

ABA in 10 µM and 100 µM concentration completely suppressed protrusion of radicle in dormant as well as non-dormant seeds (data not shown). However, the first step of the germination process was less sensitive to ABA and prominent differences between dormant and non-dormant seeds occurred. Figure 3D shows that application of 100 µM but not 10 µM ABA caused a delay of endosperm rupture of non-dormant seeds but did not affect the extent of this process. On the other hand dormant seeds showed much lower rate of endosperm rupture which was attenuated with 10 µM ABA and totally arrested with 100 µM.

In case of low concentration of KCN, PFC and SNP (10 µM) as well as in case of nitrite (500 µM and 1000 µM) the influence of dark treatment was observed. Fig. 4A and 4B shows that germination was postponed in case of dark imbibition. Seeds imbibed on light germinated earlier than seeds imbibed in dark. This effect was not visible when higher concentrations (100 µM

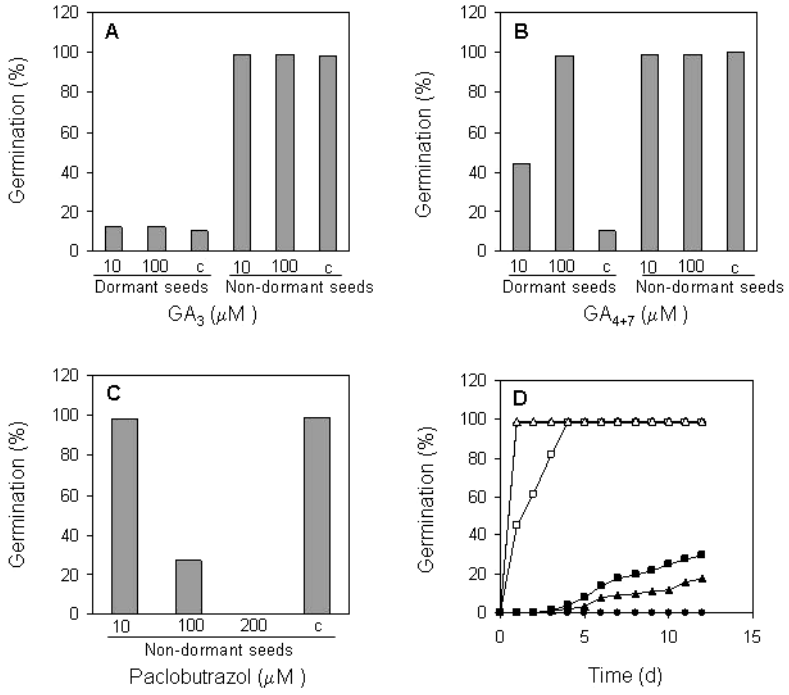

Fig. 3. Influence of giberellins, paclobutrazol and abscisic acid on seeds of *N. benthamiana*. Germination scored 12 days after the end of imbibition (A,B,C) or rupture of testa was evaluated (D).
(A) GA3, (B) GA4+7, (C) paclobutrazol, c indicates control parallel in (A), (B) and (C); (D) ABA: testa rupture: ▲ dormant seeds influenced with 10 µM ABA, ◆ dormant seeds influenced with 100 µM ABA, ■ dormant seeds-control, ◇ non-dormant seeds influenced with 10 µM ABA, □ non-dormant seeds influenced with 100 µM ABA, △ non-dormant seeds-control.

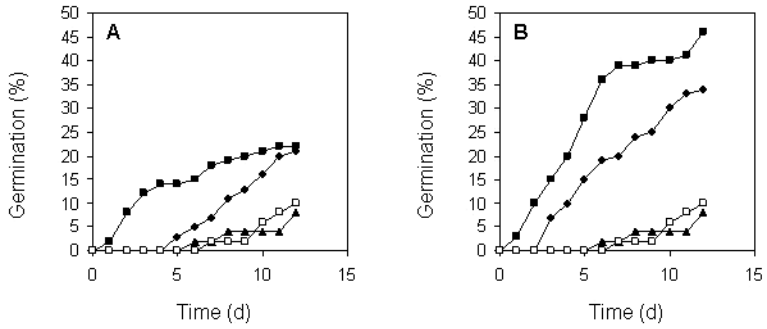

Fig. 4. Influence of light and darkness on dormancy breakage of *N. benthamiana* seeds by nitrogen containing compounds: (A) Seeds imbibed ■ 10 μM SNP on light, ▲ water on light, ◆ 10 μM SNP in darkness and □ water in darkness. 10 μM KCN or 10 μM PFC imbibition carried out in parallel gave same results as 10 μM SNP. These data were ommited for clarity. (B) Seeds imbibed ■ 1000 μM nitrite on light, ▲ water on light, ◆ 1000 μM nitrite in darkness and □ water in darkness. 500 μM nitrite imbibition carried out in parallel gave similar results as 1000 μM nitrite so these data were ommited for clarity.

and 250 μM) of KCN, PFC and SNP were applied (see above) probably due to their toxic effect.

Discussion

Seed dormancy is common in wild plants, where it may ensure the ability of a species to survive unfavorable conditions, decrease competition between individuals of the same species, or prevent germination out of season. However, it is not desired in agriculture (Finkelstein et al. 2008). We used an interesting species *Nicotiana benthamiana* in our trials because *N. benthamiana* can be used for transformations, has a short life cycle, is easy to grow and the dormancy is probably established at high temperature.

First of all we studied dormancy release due to after-ripening (Fig. 1). It is an important natural phenomenon releasing seeds of some species from dormacy. Seeds of *N. benthamiana* are strongly influenced by after-ripening. The process is quick (few weeks), mainly in case of room temperature (Fig. 1A), compare to species such as *Descurainia sophia* (one year) (Li et al. 2005). Our result suggests that after-ripening could be common among *Nicotiana* species because it was observed in case of many tobacco (*Nicotiana tabaccum*) cultivars where it is also fast (Leubner-Metzger & Meins 2000).

Mentioned process runs on molecular level, though seeds are a relatively dry system, changes in gene expression were observed (Finch-Savage & Leubner-Metzger 2007; Finkelstein et al. 2008). It was shown that after-ripening is typical for plants growing in areas with dry climate (Probert 2000) what correspond with an origin of *N. benthamiana*. It is a native species to Australia growing in dry hot areas (Burbridge 1960; Horton 1981). This could also explain why after-ripening is more efficient at higher temperature (Fig. 1A).

Second of all the influence of SNP, PFC and KCN on dormancy release was studied (Fig. 2). SNP is a photolabile molecule which releases NO so it is used as a NO donor (Giba et al. 1998; Beligni & Lamattina 2000; Kopyra & Gwózd 2003; Bethke et al. 2004a; Li et al. 2005). This donor is often employed because of its low price and stability during storage (Bethke et al. 2006). However, SNP produces cyanide (Boullerne et al. 1999; Libourel et al. 2006) which can influence the experimental system. It was shown that SNP promotes germination of wide range of species such as *Lactuca sativa* L. (Giba et al. 1998), *Lupinus luteus* (Beligni & Lamattina 2000), *Paulownia tomentosa* (Kopyra & Gwózd 2003), *Arabidopsis thaliana* (Bethke et al. 2004a), *Descurania sophia* (Li et al. 2005) and *Panicum virgatum* (Sarath et al. 2006) in NO dependent manner. Recently has been shown on *Arabidopsis* seeds that cyanide, not NO, is responsible for enhancing germination (Bethke et al 2006). So we used an analog PFC, which releases only cyanide, and is as effective as SNP in breaking dormancy of *Arabidopsis* seeds (Bethke et al. 2006). Also KCN, a long known germination enhancing compound, was used in our trials. Thus we were able to see whether SNP enhanced germination the same way as PFC and KCN. The percentage of germinating seeds, in case of SNP, PFC and KCN imbibed seeds was very similar. Our data indicate the prominent role of cyanide in breaking dormancy of *N. benthamiana* seeds as PFC induced similar dynamics and extent of germination as SNP as well as KCN.

Among other nitrogen containing inorganic compounds nitrite is known to release seeds from dormancy as well so we tested its influence at *N. benthamiana* seeds (Fig. 2).

Germination of half dormant seeds of *N. benthamiana* was enhanced by application of nitrite. Such enhancement of germination was found out in *Arabidopsis* and other species too (Bewley 1997; Bethke et al. 2006). It is thought that this compound possibly promotes dormancy release as a signal of nitrogen availability. The mode of action is still not well understood but it may act as a source of NO (Bethke et al. 2004b).

Furthermore GAs were applied to *N. benthamiana* seeds to observe whether the seed dormancy will be broken. Although it is well known that GAs are essential hormones that promote seed germination (Koornneef & van der Veen 1980; Finkelstein et al. 2008) not all known GAs are bioactive forms (Crozier et al. 2000)

and do not have the same influence in different species on dormancy breakage (Crozier et al. 2000; Li et al. 2005).

In case of *N. benthamiana* GA_3 did not break seed dormancy (Fig. 3A). Unlike GA_3, GA_{4+7} had a sought-after effect (Fig. 3B). The observed outcome is probably due to GA_4 which has already been shown to be able to break seed dormancy (Li et al. 2005). However GA_7 is considered as biologically active but its function has not been defined in regard to seed dormancy (Finkelstein et al. 2008).

In order to verify the essential role of GAs in dormancy release of *N. benthamiana* seeds paclobutrazol was applied. Paclobutrazol is a compound which lowers the content of GAs as it inhibits *ent*-kaurene oxidase which is an essential enzyme in GAs synthesis (Swan et al. 2005). It has been applied extensively in order to elucidate GAs dependency of development-related processes in plants: e.g. tuberization (Šimko 1991) and seed dormancy (Li et al. 2005). In case of *Arabidopsis* seeds application of paclobutrazol results in impaired germination (Nambara et al. 1991). Our experiments showed that germination of non-dormant seeds of *N. benthamiana* was indeed inhibited with paclobutrazol (Fig. 3C). It confirms GAs as essential hormone for seed germination in *N. benthamiana* and GA biosynthesis during germination.

We continued our trials with another important plant hormone – ABA. The treatment with exogenously applied ABA resulted in failure of germination of *N. benthamiana* seeds regardless of their dormancy state. Since germination consists of two events: rupture of testa and radicle protrusion (Arcila & Mohapatra 1983) the state of testa was also observed. We have found that ABA did not inhibit testa rupture of non-dormant seeds but partly (10 μM) or completely (100 μM) inhibited rupture of testa of dormant seeds (Fig 3D). It may be due to higher intrinsic content of ABA in dormant seeds. Similar results were obtained by Leubner-Metzger et al. (1995, 1998) with *N. tabaccum* seeds. Our finding that ABA can inhibit radicle protrusion but not first step of germination – rupture of testa in non-dormant seeds provide further support to current view on ABA that exogenously supplied cannot induce dormancy (Leubner-Metzger et al. 1995, 1998; Finkelstein et al. 2008).

Following results deal with an important topic whether the light is a necessary factor for germination of *N. benthamiana* seeds. Our experiments show that seeds of *N. benthamiana* do not germinate in darkness as well as photodormant *Nicotiana tabaccum* seeds. It is known that photodormancy of tobacco is established during seed development and can be broken by application of GAs (Leubner-Metzger & Meins 2000). Photodormancy of *N. benthamiana* dormant seeds was successfully broken by application of GA_{4+7}, but not GA_3.

It was observed that when 10 μM SNP, PFC and KCN (Fig. 4A) or nitrite (Fig. 4B) is applied on dormant *N. benthamiana* seeds there is a difference in a start of germination between dark and light imbibed seeds. Dark imbibed seeds germinated later than light imbibed seeds probably due to importance of light for germination of *N. benthamiana* seeds (photodormancy). Combined effect of light and high concentration (100 and 250 μM) of KCN, PFC and SNP did not speed up the germination compared to the same treatment in darkness. This effect is probably because of toxic effect of cyanide moiety of these compounds as germination of non-dormant seeds is inhibited in a presence of 100 and 250 μM KCN, PFC and SNP (data not shown).

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