Role of nitric oxide donors and red light irradiation on seed germination and endogenous hormones of lambsquarter (*Chenopodium album* L.)

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Abstract

Nitric oxide and red light are considered as effective signal substances for breaking seed dormancy in many plants. In this study, nitric oxide donors and red light were thus applied to seeds of lambsquarter (*Chenopodium album* L.), an important medicinal plant used frequently in traditional medicine, in order to evaluate their efficacy in breaking seed dormancy. It was observed that NO donors, especially nitrite acid (HNO_2) and sodium nitroprusside (SNP) strongly promoted seed germination of lambsquarter. Under dark condition, 10mM HNO_2 and 50-500μM SNP significantly increased germination. Germination percentage of seeds irradiated with red-light after 12 h of imbibitions in acidified nitrite (HNO_2) was significantly higher when such seeds were treated with 1-10 mM HNO_2 or 50-500 μM SNP as compared to control. In all instances synergistic application of chemicals and red-light greatly favored germination than single application of either NO donors or red light. No significant effect of potassium ferricyanide (Fe(III)CN) and potassium ferrocyanide (Fe(II)CN) on enhancing seed germination of *C. album* was observed. Bioactive gibberellins (GA_1, GA_3, GA_4 and GA_7) analysis of HNO_2 imbibed seeds showed that application of 10 mM HNO_2 and red-light to such seeds readily increased GA_4 and GA_7 levels as compared to GA_1 and GA_3 that were not significantly affected. The ABA content of seeds treated with HNO_2 and red-light also significantly reduced as compared to non treated seeds.

Media summary

Present study suggests that HNO_2 and SNP are the most effective NO donors responsible for breaking seed dormancy in lamdsquarter. GA_4 and GA_7 are main bioactive gibberellins involved in breaking seed dormancy, while HNO_2 and red-light influenced seed dormancy by significantly reducing ABA content of seeds.

Key words

Nitric oxide, red light, *Chenopodium album*, gibberellins, abscisic acid, seed germination

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Introduction

Germination process starts with the absorption of water by the quiescent seed and ends when the embryo axis breaks through the seed coats. However, in some instances, the seeds fail to germinate even when the environmental conditions for germination are favourable. Failure to germinate when environmental conditions are adequate is called dormancy (Bewley 1997; Foley 2001). Dormancy is an adaptive trait common to many plants of temperate regions. The extent and persistence of dormancy is genetically controlled and highly dependent on environmental conditions before and after seed maturation. Dormancy can be broken by a variety of signals including light (Casal and sanchez 1998), cool temperatures, stratification (Bewley 1997), hormones (Koornneef *et al*. 2002) and chemicals as widely divergent as nitrate, nitrite,cyanide (taylorson and Hendricks 1977). There is widespread agreement that nitric oxide (NO) is a key signaling molecule in plants, regulating cell function at almost all stages of development (lamattina *et al*. 2003; Neill *et al*. 2003; Wendehenne *et al*. 2004). Gemination is stimulated in a range of species by NO donors (Bethke et al. 2004). Sodium nitroprusside (SNP), potassium ferricyanide (Fe(III)CN), potassium ferrocyanide (Fe(II)CN) and acidified nitrite (HNO_2) were used as NO donors in many studies(Bethke et al. 2006; Kopura and Gwódź 2003; Bethke *et al.* 2006).

It has been well documented that abscisic acid (ABA) plays an important role in the inhibition of seed germination, whereas gibberellins (GA) is required for the induction of seed germination. Light is a critical environmental determinant for seed germination in some small-seeded plants. The effect of light on seed germination is primarily mediated by the red and far-red light, which the former promotes and the latter reversibly inhibits germination (Shinomura *et al*., 1995, 1996).

Lambsquarter (Chenopodium *album* L.) is used as vegetable and also as medical plant. Like many other wild plants, seed dormancy is highly prevalent in lambsquarter, as only a few seeds germinate after maturation. In the present study, different recommended nitric oxide donors were used to compare their ability in stimulation seed germination of dormant seeds under red light exposure and darkness. ABA and bioactive gibberellins were also measured in order to get a clear understanding of the roles of these hormones in dormancy.

Methodology

Germination test

Seeds of lambsquarters were collected from a fallow field in Kyungpook National University, Daegu, South Korea. The seeds were dried for one week at room temperature, cleaned to approximately 70% purity and sealed in transparent polystyrene containers at 4°C in a dark room. The seeds were then surface sterilized by immersion in 0.5% (w/v) NaClO for 5 min and washed with sterile distilled water. The seeds were placed in 9 cm diameter Petri-dishes with two Watman #2 filter papers moistened by 4 ml of treatment solutions, using autoclaved distilled water and analytical grade chemicals and wrapped the Petri dishes in an aluminum foil. Light emitting diode (LED) lamps with irradiation of 660 nm, 80 μ mol.m^{-2} s^{-1} were used as light source for seed germination through out the course of experiment. The experimental set up was done under very dim green light (maximum florescence of $4\times10^{-6}\mu$ mol.m^{-2} s^{-1}). All subsequent manipulations were carried out in complete darkness. Seeds germination was observed one week after treatment and emergence of radicle (2 mm) was the criterion for germination (Andersson *et al*. 2002). The experiment comprised of multiple treatments, 3 replicates of 50 seeds each per treatment. The experiment conducted was repeated three times.

Extraction and Quantification of endogenous bioactive gibberellins

The endogenous GAs levels were quantified according to the protocol of Lee *et al.* (1998). GAs were quantified with [17, 17-$^2H_2$] – GA_1, GA_3, GA_4 and GA_7 (20 ng each) as internal standards (obtained from Prof. Lewis N. Mander, Australian National University, Canberra, Australia). Major three prominent ions were analyzed by GC-MS-SIM (6890N network GC system, and 5973 network mass selective detector; Agilent Technologies, Palo Alto, CA, USA) dwell times of 100 ms. The contents of endogenous GA_1, GA_3, GA_4 and GA_7 were calculated from the peak area ratios of 508/506, 506/504, 286/284 and 224/222, respectively.

Extraction and Quantification of endogenous ABA

The endogenous ABA contents were quantified following the method of Qi *et al*., (1998) and Kamboj et al., (1999). Plant samples were extracted with 30 ml of extraction solution containing 95 % isopropanol, 5 % glacial acetic acid, and 100 ng of [(±)-3,5,5,7,7,7-d6]- ABA. The filtrate was concentrated by a rotary evaporator. The residue was dissolved in 4 ml of 1 N NaOH solution, and then washed three times with 3 ml of methylene chloride to remove lipophilic materials. The aqueous phase was brought to approximately pH 3.5 with 6 N HCl and partitioned three times into ethyl acetate (EtOAc). EtOAc extracts were then combined and evaporated. The dried residue was dissolved in phosphate buffer (pH 8.0) and then run through a polyvinylpolypyrrolidone (PVPP) column. The phosphate buffer was adjusted to pH 3.5 with 6 n HCl and partitioned three times into EtOAc. EtOAc extracts were combined again and evaporated. The residue was dissolved in dichloromethane, and passed through a silica cartridge (Sep-Pak; Water Associates, Milford, Massachusetts, USA) which was pre-washed with 10 ml of diethyl ether: methanol (3 : 2, v/v) and 10 ml of dichloromethane. ABA was recovered from the cartridge by elution with 10 ml of diethyl ether:methanol (3 : 2, v/v). The extracts were dried and methylated by adding diazomethane for GC-MS-SIM (6890N network GC system, and 5973 network mass selective detector; Agilent Technologies, Palo Alto, CA, USA) analysis. For quantification, the Lab-Base (ThermoQuset, Manchester, UK) data system software was used to monitor responses to ions of m/e 162 and 190 for Me-ABA and 166 and 194 for Me-[2H6]-ABA.

Results

Our findings revealed that germination of C. album was greatly enhanced by the application of NO donors and red light exposure. The germination rate increased with an increase in the concentration of NO donors but start declining at certain critical level. The red light pulse increased seed germination in all cases but the extent of increase varied with the concentration of NO donors. SNP produced highest germination rates as compared to other NO donors both under dark and red light regime. In case of Fe (II)CN, maximum germination 8.67% was recorded for the seeds treated with 100 uM concentration. However, 50uM Fe(II)CN plus red light pulse of 10 minutes induced 16.67% of germination. This suggest that red light is more effective at lower Fe(II)CN concentration. The germination rates increased in seeds treated with Fe(III)CN, but start reducing at 100uM and beyond. However, incase of SNP, the germination enhaced with an increase in the concentration of this NO donor, although best result of 28.67% was obtained for combined treatments of 100um SNP and red light pulse. HNO_2 also greatly promoted germination in *C. album* seeds as germination of 8% was obtained in treatments applied with 10 mM HNO_2, although maximum germination of 26.67% was observed in seeds that received both 5 mM HNO_2 and red light pulse. Germination was not observed in treatments that were subjected to 25 mM HNO_2 (see Fig 1).

These results clearly demonstrated that NO donors and red light are important for germination induction in *C. album*, but the strength of NO donors solutions demonstrate the extent of germination and efficiency of red light is also different at various NO donors amounts.

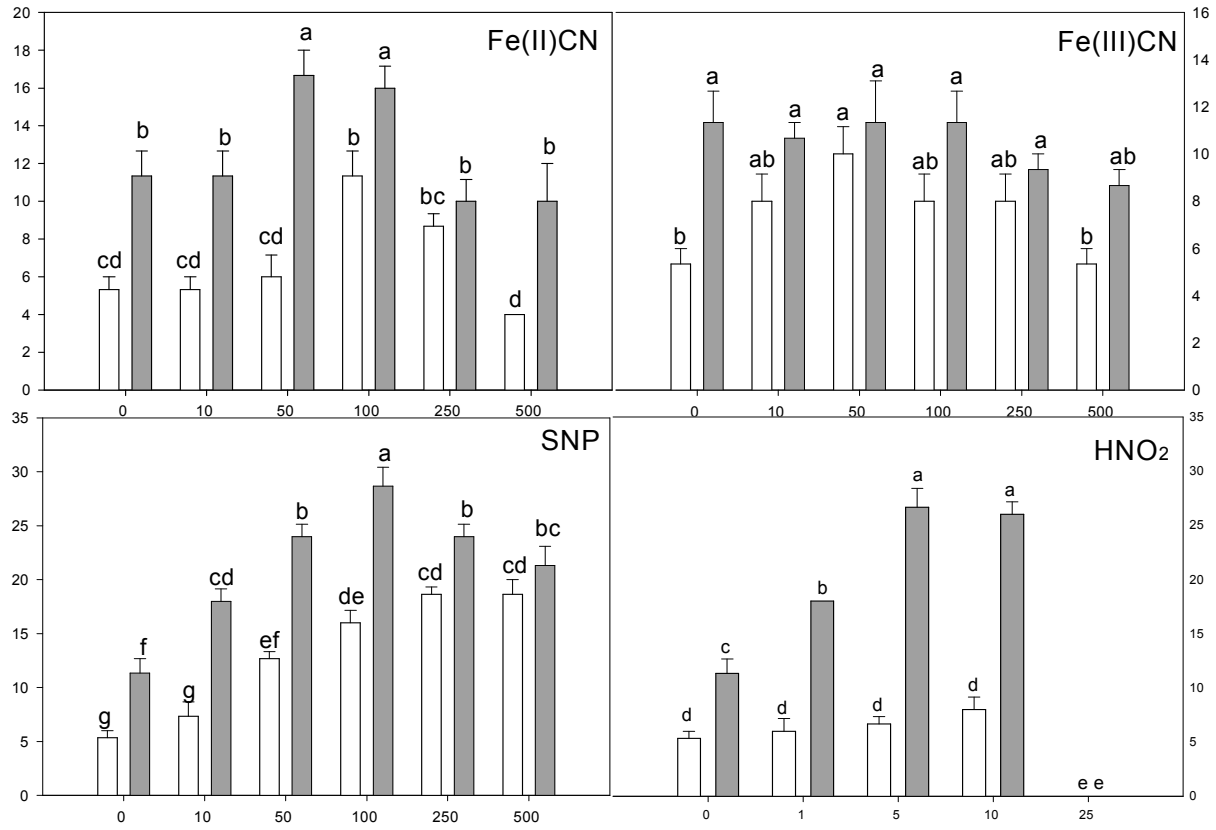

Figure 1. Action of different concentrations of NO donors on germination of lambsquarter under dark and red light regime. The white columns represent dark treatments while the black columns showed red light expousre. The concentrations of Fe(II)CN, Fe(III)CN and SNP was in µM, while that of HNO_2 was in mM.

Abscisic acid also play a pivotal role in inducing seed dormancy in many plants. Results of present study showed that ABA level decreased in seeds that were treated with HNO_2 as compared to control treatments. The lowest level of endogenous ABA content was recorded for seeds that received an incubation period of 24 hr irrespective of presence or absence of red light pulse. This clearly suggests that dark incubation period of seeds is effective in inducing higher germination percentages in *C. album* seeds (see Fig 2).

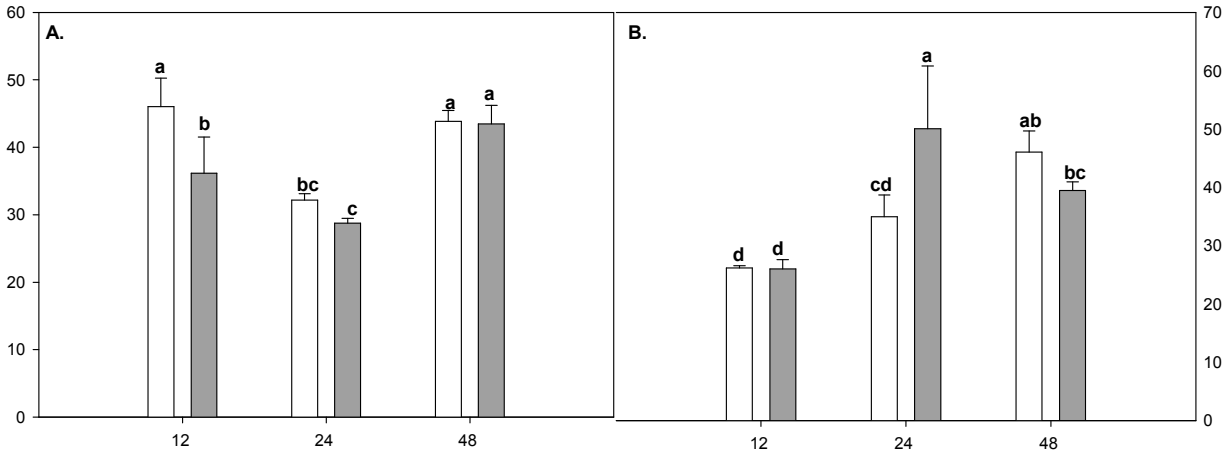

Figure 2. (A) Level of ABA (ng/g) of seeds treated with HNO_2 under 12 h, 24 h and 48 h in dark condition. (B) Level of ABA in seeds treated with HNO_2 and exposed to red light pulse of 10 minutes after 12 h of dark incubation. After red light exposure the seeds are either immediately analyzed for ABA content or placed in dark for further 12 h and 36 h. The white columns represent control while the black columns represent HNO_2 treatments.

Bioactive gibberellins aid in breaking seed dormancy in many plants. In our experiment, GA_1 and GA_3 level was higher in control treatments under dark conditions as compared to HNO_2 or red light exposed seeds. However, GA_4 and GA_7 contents increased in seeds that were treated with HNO_2 or redlight. The combined treatment of red light and HNO_2 give highest amounts of bioactive GA_4 and GA_7 (see Fig. 3). Our results suggest that GA_4 and GA_7 are more effective in releasing seed dormancy in lambsquarter as compared to GA_1 and GA_3.

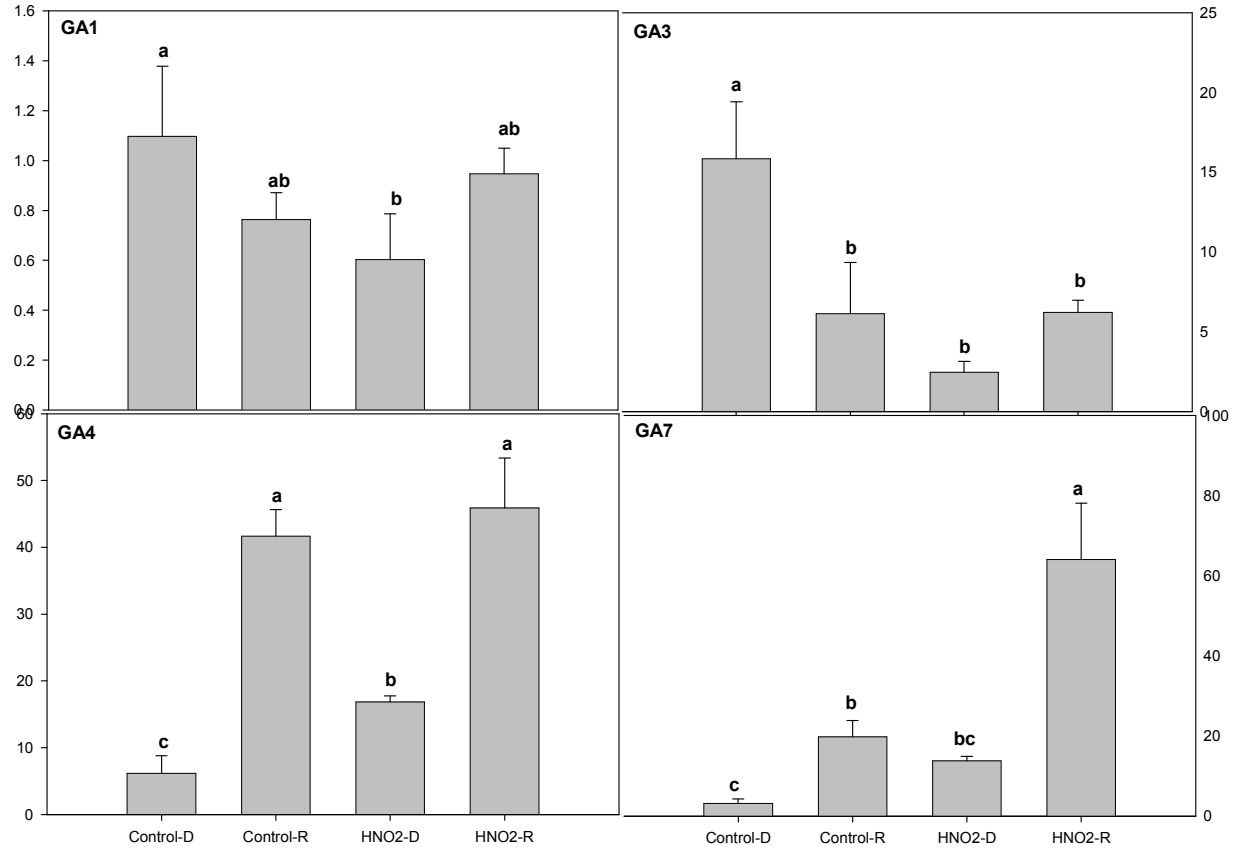

Figure 3. Level of bioactive gibberellins (ng/g) of control and HNO_2 treated seeds that are either exposed to red light or kept under dark condition. D represents darkness while R represents 10 minutes red light pulse of after 12 h of dark incubation.

Conclusion

Seed dormancy is a physiological phenomenon and breaking of seed dormancy is of immense importance in seeds with low germination potential. During present study, we tried to sort out the role of red light, NO donors and dark regime on seed germination, ABA and bioactive gibberellins. Our result suggests that SNP and HNO_2 were most effective in beaking seed dormancy of lambsquarter seeds. Red light also improved seed germination but was more effective in the presence of NO donors. Both NO donors and HNO_2 applied seeds showed reduced levels of ABA, thus confirming their positive role in loss of seed dormancy. Gibberellin analysis demonstrated that level of GA_4 and GA_7 were most favorably effected by red light and

HNO_2, thus realising the importance of these two hormones in the promotion of germination in C. album seeds.

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