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A COMPARABLE STIMULATION OF SEED GERMINATION BY GIBBERELLIC ACID AND SODIUM NITROPRUSSIDE IN *HYOSCYAMUS NIGER* L., A THREATENED MEDICINAL HERB, FROM SPITI (HIMACHAL PRADESH)

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The seeds of *Hyoscyamus niger* L. (Solanaceae), an endangered medicinal plant species from Spiti in cold desert region of trans-Himalaya, exhibited dormancy. The seed pre-treatment with sodium nitroprusside (SNP), a donor of nitric oxide (NO), led to a strong stimulation of seed germination that was comparable to that induced by GA_3. Thus, an 85 and 100% seed germination was evident due to 10 mM SNP and 1 mM GA_3, respectively. The values of other germination characteristics such as emergence index and germination speed are generally consistent with the enhancement of germination performance. Both SNP and GA_3 rapidly enhanced the α-amylase activity, lipid peroxidation level and proline contents and reduced phenolic contents in the seeds prior to the initiation of germination. The findings inter alia signify the role of reserve hydrolysis, ROS and inhibitor breakdown in the observed germination enhancement. In view of a remarkable similarity of the effects, an interplay between GA and NO in the regulation of *H. niger* seed germination is likely. KNO_3 and proline affected the seed germination only to a minor extent. The findings have implications for multiplication of *H. niger*.

Keywords: Endangered, Gibberellic acid, *Hyoscyamus niger,* Nitric oxide, Seed dormancy, Sodium nitroprusside.

Hyoscyamus niger L. (Solanaceae), Black henbane (English) or Khurasani ajwayan (vernacular), holds significant economic importance for its utilization both in classic and modern health care systems. The plant has been reported to possess anodyne, antiallergic, antiasthmatic, antidepressant, antiseptic, antioxidant, anticancer, Ca^{2+}channel-blocking, cardioprotective, hepatoprotective, mydriatic, narcotic and sedative effects (Chevallier 1996, Chauhan 1999, Alizadeh *et al.* 2014, Al-Snafi, 2018). These properties are ascribed to the abundance of an array of phytochemicals including atropine, belladonine, hyoscine, hyoscyamin, hyoscypikrin and scopolamine. Besides, *H. niger* has been employed as a model plant in diverse photomorphogenetic/ physiological studies (Robert *et al.* 1982, Taiz and Zeiger 2010).

H. niger has an endangered population status in Spiti (Himachal Pradesh, India) that comes under 'rain shadow zone' and is a part of cold desert in trans-Himalaya with typically poor and sparse vegetation. Over-exploitation and habitat destruction inter alia constitute the reasons for decline in its population necessitating the development of appropriate conservation/propagation strategies (Ved *et al.* 2003). Seeds, the principal and most convenient means of plant propagation/ multiplication, have been reported to possess dormancy in *H. niger* (Cirak *et al.* 2004, Sharma *et al.* 2006, Verma *et al.* 2014). Seed dormancy is a pause in the plant life cycle that allows germination to occur only under favourable environmental conditions and is genetically determined. Genetic evidences strongly support the central role of abscisic acid (ABA) and gibberellic acid (GA) in the maintenance and release of seed dormancy (Baskin and Baskin 2014). The effectiveness of GA_3 in dormancy release/germination improvement has been reported in certain populations of *H. niger* (Cirak *et al.* 2004, Sharma *et al.* 2006, Sharma and Sharma 2010). Nitric oxide (NO), a key signaling molecule

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(Bethke *et al.* 2004), has been shown to have a significant role in the regulation of seed dormancy and germination of several plant species including lettuce (Beligni and Lamattina 2000), arabidopsis, barley (Bethke *et al.* 2004, 2006), grass spp. (Sarath *et al.* 2006) and apple (Gniazdowska *et al.* 2010). NO effects on seed dormancy release/germination improvement have been reported to be a consequence of multiple molecular influences such as altered ABA metabolism, enhanced ethylene emission and cellular ROS management (Arc *et al.* 2013, Sami *et al.* 2019, Venkatesan *et al.* 2020, Sun *et al.* 2021). Here, we report an effective release of the dormancy in the seeds of a *H. niger* population from cold desert of Spiti in trans Himalaya by sodium nitroprusside (SNP), an NO donor. The magnitude of SNP effect was remarkably comparable to that of GA_3 with similar metabolic effects, suggesting the possibility of an interplay between GA and NO.

MATERIALS AND METHODS

Plant material: The ripened or dry capsules containing mature seeds of *H. niger* (Fig. 1) were collected from Kaza and Tabo (3400-3700 meters asl) of Spiti sub-division of Lahaul and Spiti district of Himachal Pradesh, India. The seeds were manually separated from the capsules and were air dried for at least a fortnight at room temperature and stored in airtight containers for further studies.

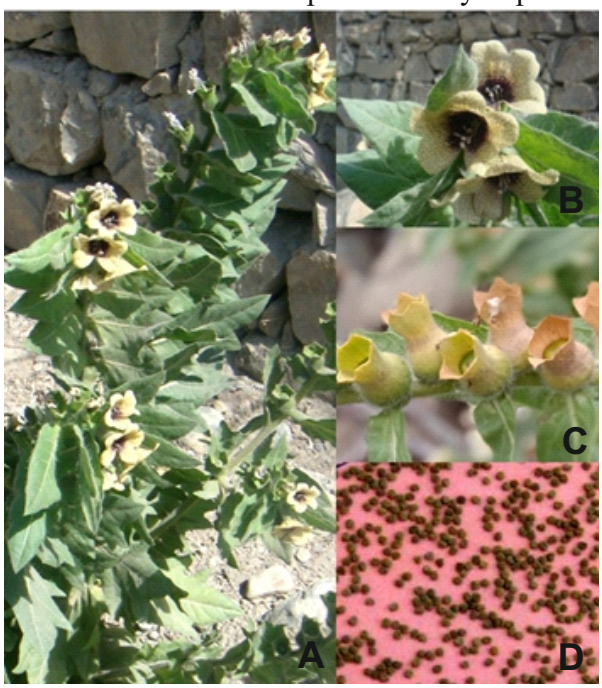

Figure 1: *Hyoscyamus niger:* whole plant **(A)**, flowering twig **(B),** mature capsules **(C)** and seeds **(D).**

Seed pre-treatments and germination: The seeds were surface sterilized with 0.1% $HgCl_2$ followed by thorough washing with water. The surface sterilized seeds were pre-treated with GA_3 (0.1 and 1 mM), sodium nitroprusside (SNP, an NO donor, 1 and 10 mM), proline (1 and 10 mM) and KNO_3 (2 mM), by keeping them submerged in test solutions for 24 h. Seeds simultaneously kept with water constituted the control. Following the pre-treatments, the seeds were transferred to Petri plates lined with two layers of filter paper moistened with distilled water and incubated in a seed germinator at 25 ± 1°C under continuous illumination (PAR: 40 µmol m^{-2} s^{-1}) provided by the fluorescent white light. The seed germination was recorded at periodic intervals until the final germination count. Emergence of 2 mm of radicle was considered as seed germination (ISTA, 1985). The experiments were performed in triplicate with 30 seeds each. In addition to the germination percentage, following parameters were calculated:

A. Emergence index (EI) =dn/n
Where, *dn* is day on which emergence ends and *n* is day when emergence starts.

B. Germination speed= $\frac{Percentage\ germination}{Day\ of\ completion\ of\ germination}$

C. Mean germination time (MGT)
The MGT was calculated according to Sharma *et al*. (2017); MGT= Σnd/N, where, n= number of seeds which germinated after each incubation period in days (d) and N= total number of seeds germinated at the end of test.

Biochemical analysis:

***α-amylase assay*:** Alpha amylase activity in the

pre-treated seeds was determined according to Filner and Varner (1967). The amount of starch degraded was calculated with the help of a calibration curve prepared with starch.

***Determination of free proline contents*:** Free proline contents in the pre-treated seeds were determined spectrophotometrically following the method of Bates *et al*. (1973). The free proline contents were determined with the help of a calibration curve.

Determination of lipid peroxidation: Lipid peroxidation in the pre-treated seeds was estimated in terms of accumulated malondialdehyde (MDA) content following the method described by Dhindsa *et al.* (1981). After homogenising and centrifuging the pretreated seeds with 0.1% trichloroacetic acid, 0.5% thiobarbituric acid in 20% trichloroacetic acid was added to the supernatant and kept at 95˚C in hot water bath for 1 h. After cooling, the absorbance was read at 532 nm. Measurement corrections for unspecific turbidity were made by subtracting the absorbance at 600 nm. The MDA contents were determined using the extinction coefficient (Heath and Parker 1968).

***Determination of total phenolic contents*:** Total phenolic contents were determined by the method given by Goldstein and Swain (1963). The pre-treated seeds were homogenised with 0.3 N HCl in methanol and the homogenate was centrifuged. The supernatant was evaporated and the residue dissolved in water that was treated with 1N Folin-Ciocalteau (FC) reagent and 35% sodium bicarbonate. After 1 h, absorbance was recorded at 630 nm and the amount of phenol contents was determined with a calibration curve prepared with gallic acid.

Protein estimation: Protein was estimated according to Bradford (1976).

Statistical analysis: All experiments were carried out in triplicate and data are presented as arithmetic means ± SE.

RESULTS

Seed dormancy, germination and responses to various pre-treatments: The seeds of *H. niger* exhibited dormancy as revealed by only 3% germination after 30 d in control. A strong stimulation of seed germination was observed due to seed pre-treatment with GA_3 and SNP. A maximum of 100 and 85% germination was evident due to 1 mM GA_3 and 10 mM SNP, respectively after 20 d. The lower tested concentrations of GA_3 (0.1 mM) and SNP (1 mM) led to only 20% germination each (Fig. 2, Table 1). Proline induced only 8% germination at 1 mM and was altogether ineffective at 10 mM. A marginal stimulation of seed germination was observed due to KNO_3 (2 mM) pretreatment (10%, 20 d). Other germination characteristics namely, emergence index and germination speed corresponded consistently with the seed germination observations. Thus, their values were highest in case of seed pre-treatment with 1 mM GA_3 and 10 mM SNP (Table 1).

Table 1: Effects of various seed pre-treatments on seeds germination, emergence index, germination speed and mean germination time (MGT) of *H. niger*. Data are arithmetic means of three replicates each.

Seed Pre-treatments	Seed Germination (%)	Emergence Index	Germination Speed	Mean Germination Time (Days)
Control	3.33 ± 0.47	1.0	0.42	1
KNO_3 (2 mM)	10 ± 2.16	2.0	1.0	7
SNP (1 mM)	20 ± 2.16	2.2	1.82	6
SNP (10 mM)	85 ± 4.24	2.0	7.08	9
GA_3 (0.1 mM)	20 ± 3.74	2.4	1.67	2
GA_3 (1 mM)	100 ± 0	5.3	14.0	4
Proline (1 mM)	8.33 ± 0.47	1.4	1.19	6
Proline (10 mM)	1.67 ± 0.47	1.0	1.0	10

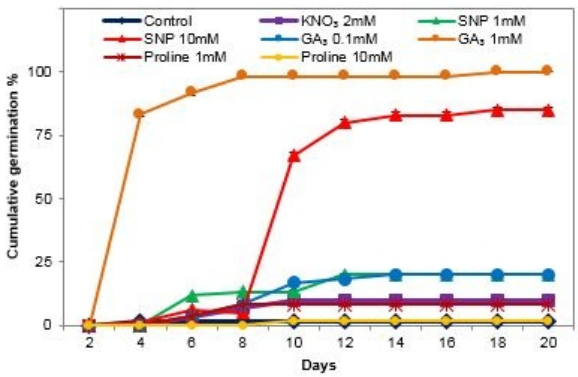

Figure 2: A time-course of seed germination of *Hyoscyamus niger* as affected by different pre-treatments. Data are arithmetic means ± S.E., n=3, each replicate comprising 30 seeds.

Biochemical changes: To get insight into the possible mechanism(s) underlying the stimulation of seed germination due to different effectors, especially SNP, certain biochemical parameters namely, *α*-amylase activity, free proline contents, lipid peroxidation levels (MDA contents) and total phenol contents were measured in the seeds after a 24 h pre-treatment.

α-amylase activity, lipid peroxidation, free proline contents and total phenolic contents: The seed pre-treatment with 1 mM GA_3 and 10 mM SNP for 24 h led to enhanced α-amylase activity in the seeds. They were responsible for 25 and 17% enhancement in α-amylase activity, respectively. The effect of KNO_3 (2 mM) and proline (1 mM) was found to be insignificant (Fig. 3A). Lipid peroxidation level (MDA contents) doubled in the seeds due to a 24 h pre-treatment with 1 mM GA_3 and 10 mM SNP. The same increased by 27 and 32% due to KNO_3 (2 mM) and proline (1 mM), respectively (Fig 3B). All tested seed pretreatments enhanced the levels of free proline in the seeds. An increase of 18, 29, 25, and 42% was observed due to GA_3 (1 mM), SNP (10 mM), KNO_3 (2 mM) and proline (1 mM), respectively in comparison to control

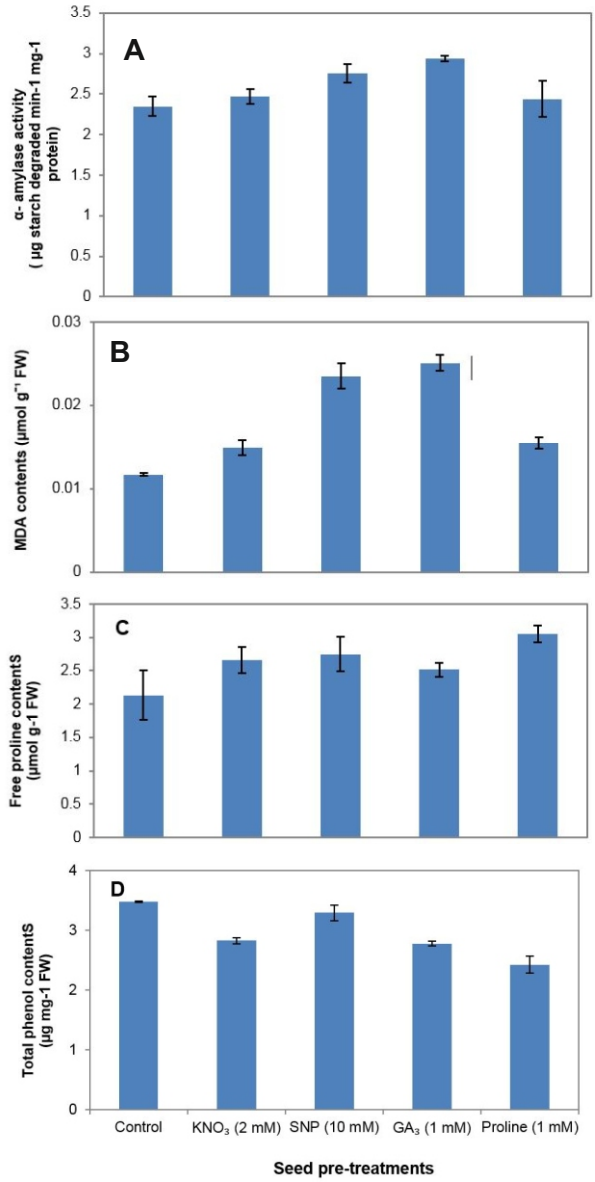

Figure 3: Effect of various seed pre-treatments on α –amylase activity (A), MDA contents (B), free proline contents (C) and total phenolic contents (D) in the seeds of *Hyoscyamus niger*. Data are arithmetic means ± S.E., n=3.

(Fig 3C). The phenolic contents of freshly harvested seeds of were 3.48 mg/g which decreased to 2.77, 3.29, 2.83 and 2.43 mg/g due to pre-treatments with GA_3 (1 mM), SNP (10 mM), KNO_3 (2 mM), and proline (1 mM), respectively (Fig. 3D).

DISCUSSION

The regulation of seed dormancy/germination relies on a combination of environmental and endogenous factors exerting both synergistic and antagonistic effects. Dormancy hampers seed germination with serious consequences for plant regeneration and eventually the productivity and yield. Its complete lack, on the other hand, is also not a desirable trait as it may lead to vivipary or pre-harvest sprouting (Bewley and Black 1994). Therefore, the management of dormancy/germination and a clearer understanding of the associated mechanisms are of paramount importance in the context of plant regeneration. In the present study, the seeds of *H. niger,* an endangered high value medicinal herb from cold desert region of trans-Himalaya, exhibited dormancy with a mere 3% germination. Aimed at overcoming the seed dormancy and improving germination, efficacy of certain commonly employed effectors such as GA_3 and KNO_3, and also of a couple of uncommon effectors namely, sodium nitroprusside (SNP, an NO donor) and proline was assessed. GA_3 expectedly proved most effective causing 100% germination at 1 mM concentration (Fig. 2, Table 1). Existence of seed dormancy and effectiveness of GA_3 in alleviation thereof has earlier been reported in *H. niger* (Radosevich *et al.* 1997, Cirak 2004, Sharma *et al.* 2006, Sharma and Sharma 2010, Verma *et al.* 2014). GA_3 involvement has been shown in the enzymatic degradation of endosperm and seed covering structure, ABA degradation or insensitivity, enhanced embryo growth potential and in turn seed germination, stimulation of cell division and also in the fulfillment of the specific requirements of after-ripening, warm or cold-stratification and light (Groot and Karssen 1987, Finkelstein *et al.* 2008, Sharma *et al.* 2010, 2014, Sharma and Sharma 2010a, Baskin and Baskin 2014, Wang *et al.* 2020). These effects likely occur concurrently and are not mutually exclusive. GA_3 induced upregulation of amylase activity in *H. niger* seeds observed here apparently facilitated the availability of low molecular weight sugars to support the stimulation of germination (Fig. 3A).

The seed pre-treatment with SNP, an NO donor, produced a qualitatively and quantitatively comparable stimulatory influence to that of GA_3 on seed germination of *H. niger*; an 85% germination was evident due to 10 mM SNP (Fig. 2). Sharma and Sharma (2010) also reported the effectiveness of SNP in seed germination of *H. niger* population from Lahaul, HP. However, the effect was much weaker than that in the present population signifying the population specificity of seed dormancy regulation and importance of population specific studies. It is quite likely that the population specific differences in response to the same effector are a consequence of variations in prevailing conditions during seed development. The efficacy of SNP as an NO donor is well known (Bethke *et al.* 2007). Its use in diverse experimental protocols is preferred in view of the inconvenience associated with the direct application of highly reactive, toxic and gaseous NO. The role of NO, a key signalling molecule, in the regulation of diverse physiological processes is unequivocally established (Arc *et al.* 2013, Sun *et al.* 2021). It has been reported to influence seed dormancy and germination in certain crop and model plant species including lettuce (Beligni and Lamattina 2000), arabidopsis, barley (Bethke *et al.* 2004, 2006, Nagel *et al.* 2019), switchgrass (Sarath *et al.* 2006), chichpea (Pandey *et al.* 2019), apple (Gniazdowska *et al.*2010, Krasuska *et al.* 2017) and *Chenopodium* (Hajihashemi *et al.* 2020). The depth of dormancy in most of the cases appeared to be shallower than that in *H. niger* seeds observed in the present study. The dormancy exhibited by *H. niger* seeds here seems to be a case of non-deep physiological dormancy according to the seed dormancy classification proposed by Baskin and Baskin (2004). Cirak *et al.* (2004) reported the existence of double dormancy (physical and physiological dormancy) in *H. niger* seeds. The physical dormancy is mostly caused by water-impermeable seed coat due to tightly packed

lignified malphigian cells, impregnated with water repellent chemicals (Sharma 2021). There was no evidence of physical dormancy in the seeds of the present *H. niger* population pointing to the population-specificity of the feature.

Furthermore, a broad similarity of the responses of α-amylase activity, lipid peroxidation and free proline levels in the seeds to GA_3 and SNP is suggestive of the reliance of the two effectors on common mechanism(s)/pathway(s). NO-dependent dormancy release has been linked with transient ROS accumulation, enhanced ethylene emission as well as ABA catabolism and lowered ABA sensitivity (Gniazdowska *et al.* 2010, Arc *et al.* 2013, 2013a, Sami *et al.* 2019, Venkatesan *et al.* 2020, Sun *et al.* 2021). NO also antagonized the blue light induced inhibition of dormant wheat seed germination (Beligni and Lamattina, 2000, Sun *et al.* 2021). The possibility of specific crosstalk between GA and NO remains to be properly understood. Nevertheless, a role of NO has been suggested upstream of GA signaling in *Arabidopsis* aleurone cells; it up-regulated a couple of genes namely, GA3ox1 and GA3ox2, involved in the biosynthesis of active GA leading to dormancy alleviation (Bethke *et al.* 2007, 2011). In contrast, NO might reduce the biosynthesis of active GAs through specific effects on GA20ox3; the latter was down regulated by NO treatment and upregulated in an NO-deficient mutant (Juste and León, 2011). A reduction in GA-dependent hypocotyl elongation due to increasing SNP concentrations was ascribed to NO induced accumulation of all DELLAs, negative regulator of GAs. Such response to NO was weaker in case of a double mutant and the insensitivity was reported to be more pronounced in the quadruple mutant 4della (Achard *et al.* 2006) and also in the 5della global knockout mutant (Juste, and León 2011). The seed dormancy release due to NO occurs is suggested to mainly involve the synthesis and perception of NO in the aleurone layer that leads to ABA catabolism/GA biosynthesis in the embryo. The GA dependent promotion of the cell wall loosening, degradation of the physical barrier allows the germination to proceed (Sun, *et al.* 2021). Besides NO, SNP also releases cyanide upon photolysis. At certain low concentrations, cyanide has been shown to regulate several plant processes including the stimulation of seed germination (Bethke *et al.* 2006a). The possibility of a joint action of NO and cyanide in SNP induced alleviation of seed dormancy in *H. niger* cannot be excluded.

The elevated MDA levels in GA_3 and SNP pretreated seeds (Fig. 3A, B) point to a role of ROS in dormancy alleviation of *H. niger* seeds. ROS generation during seed imbibition is known to have a signaling effect and has been suggested to act synergistically with NO in the release of dormancy (Bailly *et al*. 2008). Indeed, ROS and RNS concentrations falling within a defined "oxidative window" are associated with the initiation of germination; lower or greater than their desired concentration will either have no effect or cause a significant oxidative damage (Bailly *et al.* 2008). Liu *et al*. (2010) observed a rapid accumulation of H_2O_2 and NO in *Arabidopsis* seeds upon imbibition that preceded the induction of ABA catabolism/GA biosynthesis.

The seed pre-treatment with KNO_3 and proline had only a minor stimulatory influence on *H. niger* seed germination (Fig. 2). Exogenous nitrate has been suggested to have nutritional and osmotic effects on seed germination of *Avena fatua* (McIntyre *et al.* 1996). However, Alboresi *et al*. (2005) reported reduced dormancy in *Arabidopsis* seeds with impaired nitrate assimilation. Liu *et al*. (2009), employing an NO scavenger, reported that nitrate does not affect seed dormancy in *Arabidopsis* on its own but rather act through NO. Matakiadis *et al*. (2009) reported a positive regulation of gene expression of ABA catabolic enzyme by nitrate. Thus, nitrate dependent dormancy release seems to be mediated by a direct

influence on ABA metabolism and/or through an NO-dependent mechanism. Different germination characteristics such as emergence index and germination speed invariably correspond with the seed germination observations (Table 1). A decline in seed phenolic contents due to seed pre-treatments preceding the initiation of germination suggests their role in seed dormancy.

In conclusion, the findings indicate a possible interplay between NO and GA_3 in seed dormancy release of *H. niger*. A precise mechanistic basis thereof remains to be elucidated.

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