RESPONSE OF GERMINATING SEEDS OF *CICER ARIETINUM* TO 28-HOMOBRASSINOLIDE AND/OR POTASSIUM

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Summary. The involvement of ionic channel/s in signal transduction and the amplification of phytohormones induced impact on the processes leading to seed germination provoked us to undertake the present study. Seeds of *Cicer arietinum* (L.) cv. Avarodhi were soaked in the aqueous solutions of 28-homobrassinolide (HBR) and/or K^+, for a limited period, and subsequently transferred to distilled water, for the rest of the germination period. The seeds were sampled 24, 36 and 48 hours after soaking and were analyzed for relative water content, activity of nitrate reductase and for the contents of nitrate and total protein, as well. HBR treatment caused highest values of all the characteristics, except the nitrate content. Among the treatments, HBR (10^{-8}M), alone or in combination with potassium (12 μM), proved as best in activating the observed characteristics.

Keywords*:* Germination, Relative water content, Nitrate reductase activity

Abbreviations: BR-Brassinosteroid, HBR-28-homobrassinolide, NR-Nitrate reductase

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INTRODUCTION

Intracellular bodies of lipids, proteins, carbohydrates, organic phosphate and various other inorganic compounds support the process of germination of seeds and the growth of the resulting seedlings (Bewley and Black, 1985). The activation and/or synthesis of mitochondrial enzymes elevate the oxygen demand in the hydrated seeds (Salisbury and Ross, 1991). Moreover, hydrolases, lipases, proteinases and phosphatases are also released and/or synthesized (Mayer and Poljakoff-Mayber, 1989; Bernhardt *et al*., 1993) to facilitate the availability of simpler substances to the embryo, for its growth (Mayer and Poljakoff-Mayber, 1989).

Potassium is an integral part of the membrane functions (Hopkins, 1995). The ion is bound to pyruvate kinase and other essential enzymes, regulating respiration and carbohydrate metabolism (Salisbury and Ross, 1991). Moreover, potassium plays the role of *de novo* synthesis of specific enzyme proteins (Bewley and Black, 1985).

The active role of phytohormones in the regulation of seeds germination is undisputed. In addition to the five recognized phytohormones, the brassinosteroids (BRs) are also of universal occurrence in the leaves, roots and the cotyledons of the seeds (Khripach *et al*., 1999). BRs activate proton pump, thereby get involved in cell division and enlargement (Bajguz, 2000). Their role in the regulation of growth and development is thus certain (Mandava, 1988). The exogenous application of steroids, therefore, boosts seed germination in tobacco, *Arabidopsis* and wheat (Steber and McCourt, 2001; Hayat *et al*., 2003; Leubner-Metzger, 2003). BRs favour the increase in the level of carbonic anhydrase (Hayat *et al*., 2000; 2001), nitrate reductase (Hayat *et al*., 2001; Hayat and Ahmad, 2003a) and α -amylase (Hayat *et al*., 2003) enzymes. Involving gene expression (Clouse, 1997), probably makes possible the expression of the *de novo* synthesis of specific proteins (Mandava *et al*., 1988).

Seed germination is obviously a complex phenomenon, which involves finely regulated catabolic and anabolic processes where hormones and ions play a key role. The presence of steroids in the seeds provoked the authors to study its impact on some important aspects of seed germination in association with potassium, as it is required for various metabolic processes.

MATERIALS AND METHODS

Chemicals

28-homobrassinolide (HBR) was gifted by Dr. B.N. Vyas, Godrej Agrovet Ltd., Mumbai, India. All the other chemicals were purchased from Qualigens India Ltd., New Delhi, India.

Experimental design

Seeds from *Cicer arietinum* (L.) cv. Avarodhi were purchased from National Seed Corporation Ltd., New Delhi. Healthy seeds of uniform size were surface sterilized with 0.01% aqueous solution of mercuric chloride, followed by repeating washings with sterilized double-distilled water (DDW). Twenty-five seeds were soaked for 4 hours in: (i) 10^{-8} M of HBR and (ii) 10^{-6} M of HBR. These soaked seeds were wiped with filter paper and soaked for another 4 hours in (i) 6 µM of potassium and (ii) 12 µM of potassium. Seeds were soaked in another set, for 8 hours, in (i) DDW (ii) 10^{-8} M of HBR (iii) 10^{-6} M of HBR (iv) 6 µM of potassium and (v) 12 µM of potassium. Soaking duration and concentrations of HBR and potassium were based on our earlier studies (Hayat and Ahmad, 2003 b; Shad *et al*., 2004). Therefore, only two concentrations are used here.

HBR was dissolved in absolute ethanol with 5% tween-20, used as surfactant. In all the other treatments, including control, an appropriate amount of ethanol was added to obtain the required, final concentration. Each treatment was replicated five times. The experiment was repeated once. Treated seeds, after being rinsed with DDW, were transferred into sterilized petriplates, lined with absorbent cotton, moistened with 50 cm^3 of DDW only. These petriplates were incubated in dark, in a BOD incubator, maintained at a temperature of 25±2°C. Forty-five petriplates, representing each of the treatment types, including control, were placed randomly on one of the shelves of the incubator. The germinating seeds were sampled after 24, 36 and 48 hours of soaking (it also included the treatment duration of 8 hours) to assess various parameters. Protrusion of the radicle by >2 mm, outside the test, was taken as the scale for the germination, which was noted 48 hours after the soaking.

Relative water content

Seeds relative water content (RWC) was measured by determining the fractional water content (Purcell and Sinclair, 1995).

$$FWC = SFW\text{-}SDW/\ SFW$$

It was assumed that seeds at different hours of soaking were fully turgid and FWC was used to estimate the seed turgid weight (STW) for each treatment.

$$STW = SDW/(1\text{-}FWC),$$

and from this the RWC of seed was calculated:

$$RWC = SFW\text{-}SDW/STW\text{-}SDW$$

Enzyme assay

Germinating seeds (5g) were ground to a fine pulp using mortar and pestle. The pulp was resuspended in 0.1M phosphate buffer and 0.2M potassium nitrate at pH 7.5, and then was centrifuged (2000 x g) for 30 minutes. The supernatant was filtered through Whatman paper (No. 1) and stored. The activity of nitrate reductase was estimated according to Jaworski (1971), the nitrate content – according to Johnson and Ulrich (1950), and the total protein content – according to Lowry *et al*., (1951).

Statistical analysis

Treatment means were compared by analysis of variance using the statistical package SPSS. Each sampling time was analyzed separately. The data were processed by single-factor analysis of variance. LSD was calculated at 5 % level of probability.

RESULTS

Germination percentage

In all the treatments, seed germination percentage was significantly higher, than the control (Table 1). However, the lower concentration of HBR (10^{-8}M), alone or in combination to higher concentration of K^+ (12 µM), enhanced germination rate by 15% over the control.

Table 1. Individual and interaction effect of 28-homobrassinolide (HBR; 10^{-8}M or 10^{-6}M) with potassium (K^+; 6 µM or 12 µM) on the germination and relative water content percentage in *Cicer arietinum* hydrated seeds

Treatment	Duration of soaking (h)	Germination	Relative water content (%)		
			After 24 h	After 36 h	After 48 h
Control (water soaked)	8	83.5	38.8	42.2	49.2
HBR – 10^{-8}M	8	96.5	43.2	47.9	54.1
HBR – 10^{-6}M	8	92.0	39.9	43.1	50.8
K^+ - 6 µM	8	90.0	40.1	43.2	47.2
K^+ - 12 µM	8	91.5	40.6	42.9	50.5
HBR – 10^{-8}M + K^+ - 6 µM	4+4	91.0	40.5	43.0	50.1
HBR – 10^{-8}M + K^+ - 12 µM	4+4	95.5	41.2	44.1	53.7
HBR – 10^{-6}M + K^+ - 6 µM	4+4	90.5	40.4	42.8	50.1
HBR – 10^{-6}M + K^+ - 12 µM	4+4	91.0	40.6	43.1	50.6
L.S.D. P = 0.05		4.2	1.6	1.5	1.5

Relative water content

Relative seed water content improved as the germination progressed (Table 1). The HBR (10^{-8}M), alone, proved to be most effective, enhancing the values, which were the maximum throughout the assessment period, followed by HBR (10^{-8}M) and K^+ (12 µM) fed in combination.

Nitrate reductase (NR) activity

The activity of NR showed a consistent increase (Table 2), therefore, seeds, showed a maximum enzyme activity after 48 hours. Seeds, pre-treated with HBR and/or potassium, had significant increase in NR activity, at all the stages of sampling, compared to the control. A maximum increase of 61%, 77% and 109% at 24, 36 and 48 hours sampling, respectively, was noted in the seeds treated with HBR (10^{-8} M). The next lower values were recorded in the seeds where HBR (10^{-8}M) treatment was combined with K^+ (12 µM).

Table 2. Individual and interaction effect of 28-homobrassinolide (HBR; 10^{-8}M or 10^{-6}M) with potassium (K^+; 6 µM or 12 µM) on the activity of nitrate reductase in *Cicer arietinum* hydrated seeds

Treatment	Duration of soaking (h)	Nitrate reductase activity (nm NO_3 $h^{-1}g^1$f.m.)		
		After 24 h	After 36 h	After 48 h
Control (water soaked)	8	200.2	363.1	396.2
HBR – 10^{-8}M	8	323.4	642.6	831.2
HBR – 10^{-6}M	8	288.1	569.3	710.1
K^+ - 6 µM	8	235.2	419.3	560.3
K^+ - 12 µM	8	269.2	462.2	610.2
HBR – 10^{-8}M + K^+ - 6 µM	4+4	293.4	500.2	671.1
HBR – 10^{-8}M + K^+ - 12 µM	4+4	305.3	520.3	690.3
HBR – 10^{-6}M + K^+ - 6 µM	4+4	268.3	470.3	625.0
HBR – 10^{-6}M + K^+ - 12 µM	4+4	275.2	493.1	660.3
L.S.D. P = 0.05		26.5	43.4	69.5

Nitrate content

At each successive stage of germination, the nitrate content decreased, independent from the treatment (Table 3). In the control, values were significantly higher than in the treated seeds.

Protein content

On hydration, total seed protein content increased as the imbibition period was extended, being most prominent in the treated seeds (Table 3). Pre-treatment of the seeds with HBR (10^{-8}M), alone and in combination with K^+ (12 µM), generated maxi-

Table 3. Individual and interaction effect of 28-homobrassinolide (HBR; 10^{-8}M or 10^{-6}M) with potassium (K^+; 6 µM or 12 µM) on the contents of nitrate and protein in *Cicer arietinum* seeds, at 24, 36 and 48 hours of the sampling.

Treatment	Duration of soaking (h)	Nitrate (ppm)			Protein (%)		
		After 24 h	After 36 h	After 48 h	After 24 h	After 36 h	After 48 h
Control (water soaked)	8	2.2	1.6	1.4	19.9	23.0	25.2
HBR – 10^{-8}M	8	1.9	1.4	1.1	25.9	29.8	30.2
HBR – 10^{-6}M	8	2.0	1.5	1.2	21.6	24.7	28.3
K^+ - 6 µM	8	1.9	1.3	1.0	23.6	27.4	28.2
K^+ - 12 µM	8	2.0	1.4	1.1	23.1	28.0	29.0
HBR – 10^{-8}M + K^+ - 6 µM	4+4	1.9	1.3	1.1	21.6	24.7	28.0
HBR – 10^{-8}M + K^+ - 12 µM	4+4	1.9	1.4	1.2	24.9	28.3	29.9
HBR – 10^{-6}M + K^+ - 6 µM	4+4	1.8	1.3	1.1	21.6	36.1	27.1
HBR – 10^{-6}M + K^+ - 12 µM	4+4	1.9	1.3	1.1	23.4	24.9	26.8
L.S.D. P = 0.05		0.11	0.13	0.07	1.2	1.4	2.2

mum values and, in its effect, was very closely followed by HBR (10^{-6}M) + K^+ (12 µM).

DISCUSSION

Hydrophilic groups (-NH_3, -OH and/or –COOH) of proteins and carbohydrates, located in the seed coat, during imbibition, attract the dipolar water molecules to form hydrated shell around these macromolecules. This facilitates not only the swelling of the seed coat, but also makes it more permeable to oxygen and water. This natural phenomenon is obviously responsible for the increase in the water content of the seeds. However, the values for water content was quite prominent in the seeds, pretreated with HBR, alone or in association with potassium, compared to the water soaked control (Tables 1 and 2). Probably, this increase in the water content of the HBR-treated seeds is an act of the hormone on the proton pump of the cell wall (Bajguz, 2000) facilitating its loosening, thus, favoring the cellular elongation (Khripach *et al*., 1999).

Hydration of the seed is associated with the activation of the existing proteins, their hydrolysis and/or *de novo* synthesis of the required protein types (Bewley and Black, 1985). Our observation revealed that the activity of nitrate reductase (NR) increased with the progress of the germination (Table 2). This observation is in accordance with Tahir and Farooq (1989) and Hayat and Ahmad (2004). In the soaking medium, nitrate (inducer of NR) has not been supplied from outside, therefore, the increase in NR activity is at the reserve level of the nitrate in the seeds. It could be proposed that this increase in NR activity cannot simply be substrate induced (Afridi

and Hewitt, 1964) but generated by HBR alone or in association with potassium. This and other studies (Mai *et al*., 1989; Hayat *et al*., 2001; Hayat and Ahmad, 2003a) clearly demonstrate the role of phytohormones and steroids in inducing the activity of NR. Metabolic changes generated by brassinosteroids may be suggested through their impact at the level of the genes where the activity of DNA and RNA polymerases is known to increase (Kalinich *et al*., 1985). Total protein content increased (Table 3) (epibrassinolide induced quantitative and qualitative changes of soluble and insoluble proteins (Khripach *et al*., 1999) and homobrassinolide induced changes in proteins (Hayat and Ahmad, 2003b)) and provided additional support to the above concept. Mandava (1988) has studied the potentiation of new proteins synthesis, along with the steroid solubilization of the stored ones. Moreover, comparing the data of the level of NR (Table 2) to that of total protein (Table 3) in response to the treatment, a parallel relationship seem to exist, confirmed by Singh and Singh (1985).

The rate limiting step (reduction of nitrate to nitrite) in the process of reduction of nitrate to organic nitrogen (Hopkins, 1995) should have been activated under high NR activity. NR levels showed in Tables 2 and 3 suggest an inverse relationship to that of the nitrate. The availability of organic nitrogen in sufficient quantities may have supported not only the liquefaction of the stored proteins but also the synthesis of the required proteins under steroidal action (Mandava, 1988). HBR, therefore, favoured the recycling of proteins and may have regulated the orientation of microtubules (Catterou *et al*., 2000). A cumulative effect was observed in the form of extended growth of radicle and plumule, during the cellular elongation (Khripach *et al*., 1999) - marker for the germination (Table 1). It supports BRs induced stimulation in seed germination of *Arabidopsis,* wheat and tobacco (Steber and McCourt, 2001; Hayat *et al*., 2003; Leubner-Metzger, 2003).

It is evident from Tables 1 to 3 that the presence of potassium, with or without HBR, had a positive impact on the germination process of the seeds. It will be premature to make a particular conclusion from the present data although potassium is known to be the activator of many enzymes involved in photo-, starch- and protein-synthesis and respiration (Bhandal and Malik, 1988). Its presence in the soaking medium, at a level of 12 μM, has also accelerated germination and the activity of α-amylase in the seeds of *Hordeum vulgare* (Shad *et al*., 2004). However, additional studies are needed to specify the place of action of the potassium ion.

It can be concluded that seeds of *Cicer arietinum* soaked in HBR (10^{-8}M), alone or in combination with potassium (12 μM), increase organic nitrogen content, which obviously leads to a higher vigor.

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