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ORIGINAL ARTICLE

Induction of hydrolytic enzyme activities in dormant seeds of *Dracocephalum kotschyi* Boiss. causes improvement of germination and seedling vigor indices

Rezvan Ghannad[1] · Maryam Madadkar Haghjou[1] · Ali Raza[2] · Mirza Hasanuzzaman[3]

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Abstract

To overcome the seed dormancy of *Dracocephalum kotschyi* Boiss., a high-valuable herbaceous and medicinal plant, priming technique was applied using mechanical treatment (scarification, Scr) alone, or in combination with various chemical treatments under two groups viz. group-1: KNO_3, KH_2PO_4, Na_2SiO_3, and group-2: ethanol, ascorbate, gibberellin, polyethyleneglycol (PEG), and complete Johnson nutrient solution (J.N.S). Results showed that scarification increases α-amylase, β-amylase, dehydrogenase, and protease enzymes activities and consequently causes higher seed germination compared to the control. Notably, significant positive effects were observed when the joint mechanical scarification–chemical-priming was applied. Enzymes' activities and metabolite contents were increased in collective primed seeds, especially at the shorter priming times of group-1 and in Scr-GA3-Cold and SCr-J.N.S treatments of group-2. About 97–100% final germination percentage (FGP) and the first germination on 2nd–3rd day were obtained by Scr-GA3-Cold and SCr-J.N.S followed by the improvement of seedling growth indices. The highest GRI, TI, Vig (I), Vig (II), chlorophylls (Chl), the least mean germination time (MGT), and T50 indices were obtained by Scr-GA3-Cold treatment. In general, the most effective treatments for germination of seed and seedling growth improvement could be sorted as; Scr-GA3-Cold ≥ Scr-J.N.S > Scr-Ethanol, Scr-KNO_3-(3h) > Scr-Asc ≥ Scr-PEG. The joint scarification + chemical-priming induced hydrolytic enzyme activities were more effective than other treatments and caused the breakdown of *D. kotschyi* seeds dormancy. Notably, hydrolytic enzyme activities (especially amylases) could be considered an indicator for selecting the best priming treatments in advance before seed planting.

Keywords Ascorbate · Amylase · Ethanol · Chemical priming · Seed dormancy · Medicinal plants

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✉ Maryam Madadkar Haghjou
madadkar.m@lu.ac.ir

✉ Mirza Hasanuzzaman
mhzsauag@gmail.com

Rezvan Ghannad
ghannadrezvan@gmail.com

Ali Raza
alirazamughal143@gmail.com

[1] Department of Biology, Plant Physiology, Faculty of Science, Lorestan University, Khoramabad-Tehran Road (5th K), Iran

[2] Key Lab of Biology and Genetic Improvement of Oil Crops, Oil Crops Research Institute, Chinese Academy of Agricultural Sciences (CAAS), Wuhan 430062, China

[3] Department of Agronomy, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka 1207, Bangladesh

Introduction

Dracocephalum kotschyi (*D. kotschyi*), an important herbal drug used in traditional medicine because of various medical effects, such as antispasmodic and immunemodulatory properties (Sadraei et al. 2015), the biological activity of essential and volatile oils (Sadraei et al. 2015), and its antioxidant and anticancer activities (Talari et al. 2014; Moradi et al. 2020). According to Heydari et al. (2019), this valuable plant is classified as endangered plant due to low seed germination rate, extra harvesting by native peoples, and limited distribution areas. Low and erratic seed germination of *D. kotschyi* has been attributed to seed dormancy (Fattahi et al. 2011; Moradi and Otroshy 2012). Seed dormancy is a physiological phenomenon that occurs by external or internal effectors, such as rigid seed coats, immature embryos, and inhibitory materials (Estaji et al. 2012).

To solve this problem, some researchers have already worked on micro-propagation of *D. kotschyi* using tissue culture techniques (Otroshy and Moradi 2011, 2013) and its seed conservation via vitrification and encapsulation-dehydration as cryopreservation methods (Shahabfar et al. 2018). Additionally, some scientists have tried to improve seed germination and break down the dormancy of two species, i.e., *D. argunense* Fischer ex link (Chang et al., 2009) and *D. kotschyi* Boiss (Fattahi et al. 2011; Moradi and Otroshy 2012). They treated plants with various temperatures (4 °C, 15–25 °C, and 30 °C), light (darkness and 16 h photoperiod of 28–36 mmol m^{-2} s^{-1}), inorganic salts (KNO_3 and KH_2PO_4), scarification, gibberellic acid (GA3), cytokinin and indole-3-acetic acid (IAA) only as seed pre-soaking or seed culturing. However, they did not continue the procedure till re-drying the seeds.

The standard chemical seed priming method includes soaking the seeds in solutions and re-drying the seeds back to their original water content (Lutts et al. 2015). The primed seed, which is surface-dried, can be stored for even two months as a positive point. Whereas, pre-soaked seeds are partially germinated before planting, and roots and shoots have emerged from the seed coat in the more vigorous seed and must be planted wet (Brede and Brede 1989). Wet seed and seedlings could be susceptible to physical damage (Paparella et al. 2015; Shafiq et al. 2015). Seed priming has been used to apply different seed invigoration treatments and induce various activities related to seed germination, e.g., respiration, endosperm breaking down, gene transcription. These processes stimulate quiescent dry seeds to enter the germination stage and improve the germination ability. At the same time, priming imposes abiotic stress on seeds. It causes the stimulation of stress reactions, such as accumulation of late embryogenesis abundant (LEA) proteins and the induction of cross-tolerance (Chen and Arora 2013).

Evaluations of some hydrolytic enzyme activities, such as α- and β-amylase (Lu and Sharkey 2004; Kashem et al. 2013), dehydrogenase (Oaikhena et al. 2013), and protease (Padmakar et al. 2005; Ramakrishna and Ramakrishna Rao 2005), are excellent criteria for tracking the seed germination and hydrolysis of seed storage for protrusion of root. Chen and Arora (2013) documented that seeds treated by priming techniques should have higher germination power manifesting by a sooner and higher accumulation of Type II proteins, such as α-amylase and β-amylase. Amylases are very important in the hydrolysis of starch, and seeds with higher level of these enzymes facilitate the accessibility of their embryos to carbohydrates, resulted in higher seed vigor and germination rate. They are known to be significantly related to seed quality (Oliveira et al. 2013). The metabolic activation process to produce amylases has been comprehensively studied. Gibberellin causes transcription of the genes which encode α-amylase in the aleurone layer (Appleford and Lenton 1997), and secreted α-amylase in endosperm causes carbohydrates reserve mobilization, resulting in radicle elongation (Lovegrove and Hooley 2000). Dehydrogenases belong to the oxidoreductase class and are considered necessary enzymes for the respiratory process, seed viability, and germination (Kerscher et al. 2007). Other enzymes like proteases are also vital in free amino acid mobilization and protein biosynthesis in endosperm and embryo to precede seed germination (Kirmizi and Güleryüz 2006; Joshi 2018).

Enormous information from the benefits of seed priming encouraged us to use this method on *D. kotschyi* seeds to pull forward the onset of germination and increase its final germination percentage (FGP) by promoting and stimulating the metabolic germination activities in dormant seeds. Additionally, finding the best treatment for the improvement of seed germination and seedling growth was one of our vital aims. The mechanical/chemical/mechanical treatments + chemical-priming were used, and the hydrolytic enzyme activities, protein, and sugar content in re-dried seeds were assessed. Additionally, the germination trend and seedling growth indices were also evaluated. We observed high inductions in hydrolytic enzyme activities in primed seeds, together with the increases in metabolite contents caused by a combination of mechanical and chemical treatments. These were then confirmed by the achievement in germination percentage up to 100% in primed seeds. Besides, significant differences among the influences of treatments on seed germination and seedling growth have appeared.

Materials and methods

Seed source and materials

The primary seeds of *D. kotschyi* Boiss. were collected from the plants grown around the Semirom (Dowlat Quairn, 31° 31′ 59″ N, 51° 38 ′22″ E). Then the seeds were handled by specialist researchers at seed company (Pakan Seed Center, Isfahan, Iran) and planted in a small farm after a preparation process (as wetting the seeds with distilled water and keeping them in a fridge (+4 °C) for 4 weeks). This step was applied for the stimulation of seed germination. After planting, the seeds were gradually germinated for about 20–30 days, and after six months; the seeds were collected and kept at 25–27 °C at the seed center. As chilling could be a treatment, the seeds were kept at 25–27 °C after receiving them in our lab, and we tried to do the experiments in a short period for more consistency in seed lot properties. *D. kotschyi* Boiss. seeds weight was 3 g ($n = 1000$). Chemicals (analytical grade) were provided by Sigma (Germany).

Experimental details and seed priming

Mechanical scarification and chemical-priming treatments were designed, as described in Fig. 1, including scarification, halopriming [(potassium nitrate (KNO_3), monopotassium phosphate (KH_2PO_4), calcium chloride ($CaCl_2$) and Johnson medium)], osmopriming with polyethylene glycol (PEG) and hormopriming [(sodium silicate (Na_2SiO_3) and GA3)], and also ascorbate and ethanol; separately or as combined mechanical + chemical treatments.

Scarification as mechanical treatment was done by sandpaper to overcome the seed coat resistance, in a way that each seed precisely dragged four times on a 10 cm length of sandpaper.

In addition, two different groups of chemical treatments were designed. Group-1 priming treatments, were prepared according to the previous reports: 0.9% KNO_3, 1.5 mM Na_2SiO_3 (Torabi et al. 2012), 0.9% KH_2PO_4 and 0.12 M $CaCl_2$, and group-2 as: 7% ethanol, 10 mM ascorbate (Tizfahm et al. 2016), − 3.5 bar PEG (Shukla et al. 2016), 500 ppm (GA3, MW ~ 196 g mol^{-1}) together with cold temperature at 4 °C (González-López and Casquero 2014), and finally a modified Johnson nutrient solution (J.N.S) (Johnson et al. 1968; Shariati and Lilley 1994) including; KNO_3 (5 mM), $MgSO_4$ (5 mM), KH_2PO_4 (0.2 mM), $CaCl_2$ (0.2 mM),NaEDTA (5 µM), $FeCl_3$ (2 µM), $MnCl_2$ (7 µM), $ZnCl_2$ (1 µM), $CoCl_2$ (1 µM), $CuCl_2$ (1 µM), $(NH_4)_6Mo_7O_{24}$ (1 µM), $NaHCO_3$ (0.02 M).

Group-1 treatments were applied for each 3, 6, 9, and 12 h, at 16 °C, to show the priming time trend. Group-2, including more different substances, some preliminary germination experiments were performed at different priming times 1–48 h at various temperatures. The best time and temperature were chosen based on the highest FGP for the present experiments. Therefore, priming with group-2 treatments (except GA3) was applied for 3 h at 16 °C and with GA3 for 24 h at 4 °C. Concentrations of treatments were also determined either by some preliminary studies or according to previous studies as mentioned above.

The control 1 received no mechanical treatment or chemical-priming and after surface sterilization (SS), only was treated with deionized water (DIW) for each 3, 6, 9, or 12 h. The control 2 experienced only scarification before treatment with DIW. The control 3 was not subjected to scratching but was treated with chemical solutions from either group-1 or 2 (separately, at their correspondence times). In the case, controls had nearly the same or close values at different treatments (times and/or chemicals treatments), an average (± SD) was considered as single control (e.g., control 1, 2, or 3 for group-1 treatment, and control 1 or 2 for group-2).

For surface sterilization before priming, seeds were first dipped in Vitawax (0.2%) solution for 10 min. Then, four replications for group-1 and six replications for group-2 of 50 seeds (as one replication) were sown in Petri dishes (90 mm × 15 mm) having one layer of filter paper (Whatman#2) moistened with 10 mL of the mentioned solution treatments. After finishing the priming times, seeds were

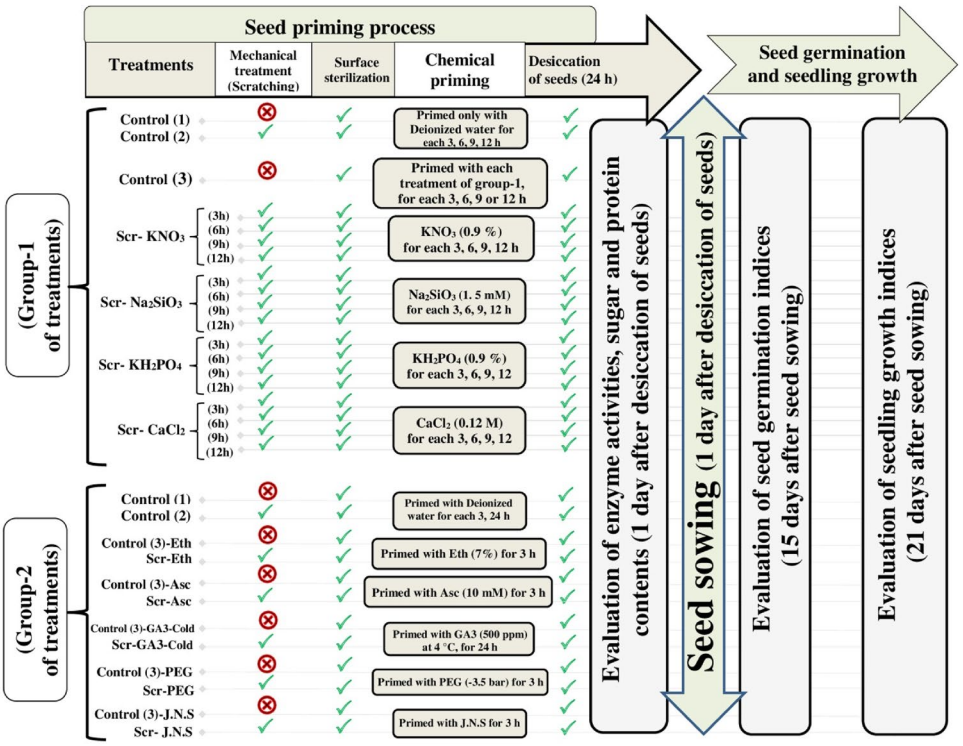

Fig. 1 Description of group-1 and group-2 priming treatment preparations (from left to right). For each control, a single average of evaluated indices at different treatments and/or different times was considered as control 1, 2 or 3. Chemical priming for all was applied at 16 °C and dark, except for GA3 treatment conducted at 4 °C. Seed desiccation to return to their initial water content was conducted at room temperature (25 °C) and dark for 24 h. *Scr* mechanical scarification, *Eth* ethanol, *Asc* ascorbate, *GA3* gibberellic acid, *PEG* polyethylene glycol, *J.N.S* Johnson nutrient solution

quickly put on a filter paper at 25 °C for 24 h to be dried back to their initial water contents. The weight of seeds was checked before and after the treatments. All priming processes were performed in the dark to avoid the unknown effects of light. Re-dried seeds, collected from this stage, were divided into two parts; the first part was used for enzymes and metabolite evaluations, and the second part was used for sowing in Petri dishes for 21 days. Seed germination was monitored till the 15th, and some of the seedling growth indices were assessed on 21st day.

Evaluation of hydrolytic enzymes activities

Evaluations of enzymatic activities and metabolite contents were performed one day after priming, and the absorbances were read by using a 96-well spectrophotometer (Model Epoch, BioTek Co., UK.)

α-amylase and β-amylase activities

The activities of α- and β-amylase were evaluated according to the method of Black et al. (1996) and Białecka and Kępczyński (2010), respectively. For extraction and homogenizing the seeds in a mortar, 20 mM phosphate buffer (pH 7.4 containing 2 mM $CaCl_2$) and 16 mM sodium acetate buffer (pH 4.8) were used, respectively in the case of α- and β-amylase evaluation. The absorbances were read at 620 for α-amylase and 540 nm for β-amylase.

Dehydrogenase activity

Seeds were mixed with Trizma base buffer (pH 7.4, containing 0.05 g of 2,3,5-triphenyl tetrazolium chloride), according to Kittock and Law (1968). Methyl Cellosolve containing 0.25 g trichloroacetic acid was used for pulverizing the seeds. The absorbance was read at 480 nm.

Protease activity

The protease activity was measured according to Ramakrishna and Ramakrishna Rao (2005) by azocasein as substrate. Seeds were ground thoroughly in a mortar with chilled 0.05 M Tris–HCl buffer (pH 7.2) and 2 mM β-mercaptoethanol. The absorbance was read at 440 nm.

Total soluble sugar, reducing sugars and protein contents evaluations

Total soluble sugar amount was measured by the Anthrone method (Irigoyen et al. 1992), and reducing sugar content was quantified using a dinitrosallcylic acid (DNSA) method from Miller (1959). Soluble protein content was assessed according to Bradford (1976). The absorbances were read at 625 nm for TSS evaluation, 540 nm for reducing sugar assay, and 595 nm for protein assay.

Germination test conditions

The second portion of re-dried seeds after priming (mentioned in 'Experimental details and seed priming'), was considered for germination tests and transferred to the Petri dishes. Similar to the priming replicates, four replicates for group-1 and six replicates for group-2 of 50 seeds (as one replication) were sown in Petri dishes (90 mm × 15 mm) with one layer of filter paper (Whatman #2) moistened with 15 mL of DIW. Petri dishes were then incubated in the following conditions; 25/18 °C (day/night), 8/16 h photoperiod, with a relative humidity of ~ 50%. The light was provided by white fluorescent Lights, intensity ~ 50 µmol m^{-2} s^{-1} measured by a photon meter (Hansatech QSPAR, UK). The number of germinated seeds with a protruded radicle length > 1 mm (de Castro et al. 2000) was monitored daily for 15 days. Growth indices, such as shoot length (SL), root length (RL), and their dry weights (SDW and RDW, respectively) in addition to the leaf pigments [(chlorophyll (Chl) and total carotenoid)], were recorded 21 days after seed sowing.

Data analysis

The indices: germination start day (GSD), final germination percentage (FGP; Kader 2005), mean germination time (MGT; Kader 2005), time to 50% germination (T_{50}; Salehzade et al. 2009), germination rate index (GRI; Hosseini et al. 2013), Timson Index (TI; Ajmal Khan and Ungar 1998); Pujol et al. 2000), Vigor (I) index; (Vig (I); Abbasian and Moemeni 2013), Vigor (II) Index (Vig (II); Abbasian and Moemeni 2013) were calculated according to the following formulas:

$$\mathrm{FGP} = \frac{\text{total number of germinated seeds}}{\text{total number of seeds}} \times 100$$

$$\mathrm{MGT} = \frac{\sum f \cdot x}{\sum f}$$

f is the germinated seeds in day x.

$$T_{50} = \frac{\left[\left(t_2 - t_1\right) \times 50\right] + \left(P_2 t_1 - P_1 t_2\right)}{P_2 - P_1}$$

Here, t_1 and t_2 are times when the germination percentage is less and more than 50%, and P_1 and P_2 are the germination percentage at t_1 and t_2, respectively.

$$\mathrm{GRI} = \frac{\sum n}{\sum dn}$$

n is the number of germinated seeds in each day/since the first day of the experiment.

$\mathrm{TI} = \Sigma G_i / T$,

G_i is the the percentage of germinated seeds in each day, T is the total number of experiment days.

Vigor (I) = (Shoot length + root length) × germination percentage

Vigor (II) = (Shoot DW + Root DW) × germination percentage

Statistical analysis

All experiments were performed as completely randomized design (CRD), and significant differences of means were separated using Duncan's test ($P < 0.05$). SPSS Version 16.0. (SPSS Inc., Chicago, USA) was used for data analysis.

Results

D. kotschyi seeds have a relatively hard seed coat and a mucilaginous shell (Fig. 2). Therefore, the mechanical scarification technique was used either alone or in combination with chemical treatments to evaluate its effects on seed dormancy, germination, and seedling growth. In this research, mechanical treatment in a uniform and the precise procedure has been reported, which is completely repeatable. Besides, the priming technique was applied instead of the direct seed pre-sowing and used several different chemicals, most of which have not already been applied to break the seed dormancy of *D. kotschyi*.

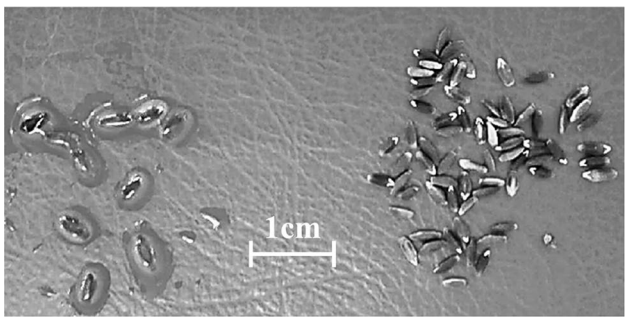

Fig. 2 *Dracocephalum kotschyi* Boiss. seeds. Wet seeds covered by a mucilage shelf (left side) and dry seeds (right side)

Seed priming treatments increased the hydrolytic enzymes activities and metabolite levels in *D. kotschyi* seeds

The comparison of enzyme activities and metabolite levels in scratched seeds (control 2) to those in non-scratched seeds (control 1) (Figs. 3, 4), showed the notable inductions of enzyme activities in mechanically treated seeds by scarification. As mentioned earlier, both control 1 and 2 were primed by DIW (hydro-priming) and then returned to their initial water content. Only control 2 was scratched before hydro-priming. The activities of α-amylase (35–38%), β-amylase (27–32%), dehydrogenase (106–117%), and protease (60–69%) were increased in control 2 over control 1. Some elevations were also observed in protein content (41–47%), total soluble sugar (25–26%), and reducing sugar contents (72–74%) (Figs. 3, 4). As shown in Fig. 3, the hydrolytic enzyme activities of α-amylase and β-amylase, and dehydrogenase (Fig. 3A–C) were significantly increased in all joint scratched-chemical primed seeds compared to the three controls 1, 2, and 3, confirming the role of joint mechanical scratching and chemical-priming. Protease activity and protein content were also responded increasingly to priming treatments (Fig. 3D, G). Likewise, protease activity and protein content were both increased in scratched seeds (Figs. 3 and 4, parts D, G). Interestingly, 12 h priming of scratched seeds by treatments of group-1, caused the highest protease activities as well as the lowest protein contents (Fig. 3). In group-2, Scr-PEG with the highest protease activity showed the lowest protein content, and the reverse condition was observed for Scr-GA3-Cold (Fig. 4).

Besides control 1 and control 2, a third control (control 3) was designed, which was not scratched but was treated by chemical treatments at the designed condition of group-1 (at either 3, 6, 9, or 12 h) or group-2 at their correspondence times (Fig. 1). Surprisingly, in seeds primed by group-1, the amounts of evaluated indices from control 3 were approximately similar or nearly close to those from control 1 (Fig. 3, Tables 1, 2, 3). These results suggest that treating the seeds with only chemical substances has no major effect as much as scratching on metabolism activation. Further, group-2 treatments positively influences the enzyme activities, seed germination, and seedling growth compared to group-1 (Fig. 4, Tables 1, 2, 3).

Moreover, the positive effects of scarification on the activation of metabolism in mechanically scratched seeds were continued as an improvement of FGP from zero in non-scratched seeds (control 1) to 34–36% in scratched seeds (control 2) during 15 d of seed soaking in Petri dishes (Table 1). On the other hand, the chemical-priming accompanied by mechanical treatment may positively affect physiological events in seeds, which encouraged us to treat scratched seeds by each KNO_3, Na_2SiO_3, KH_2PO_4,

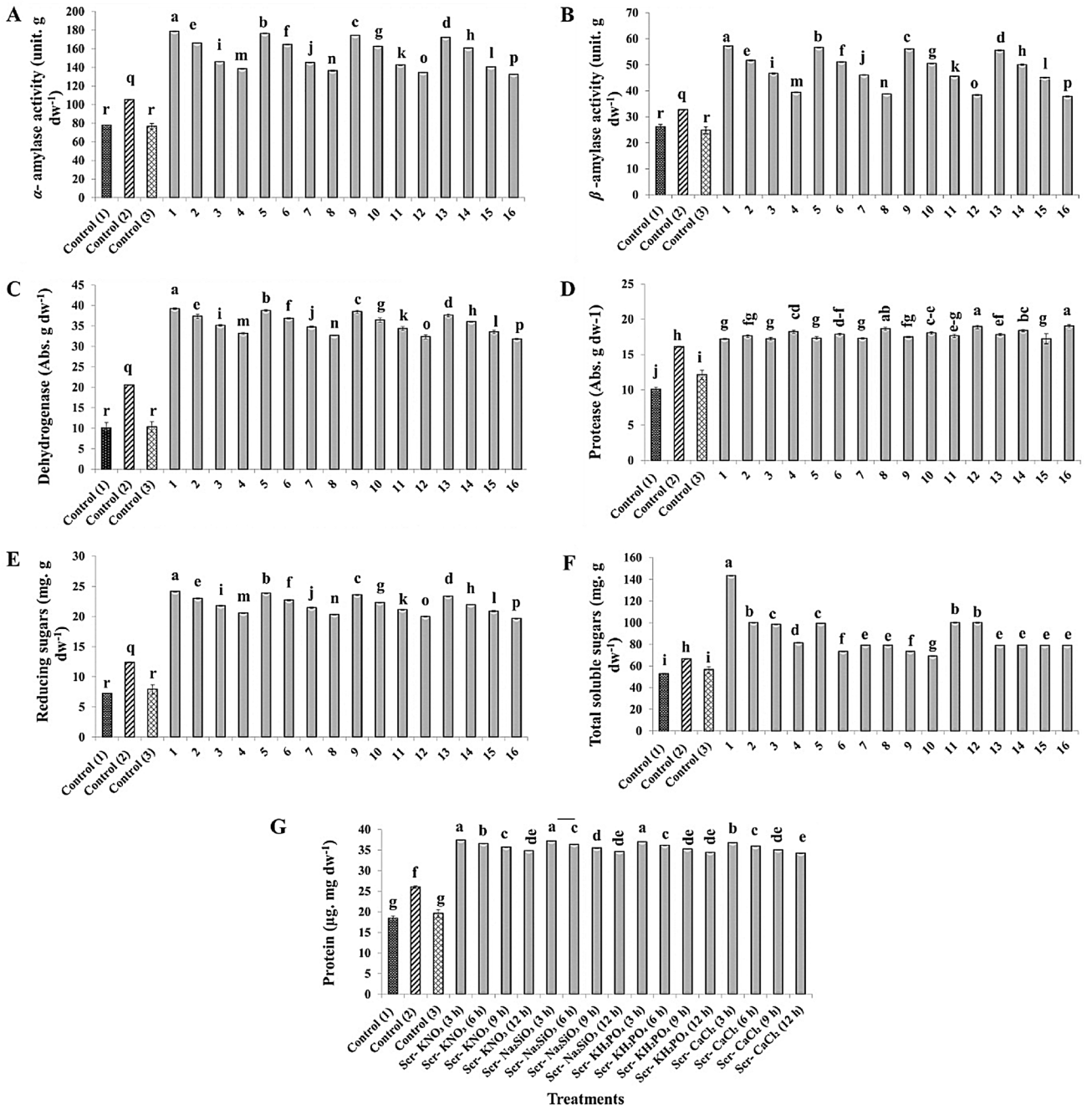

Fig. 3 Enzyme activities, total soluble sugar and protein content of *D. kotschyi* Boiss. seeds one day after priming with group-1 of treatments including; KNO_3, Na_2SiO_3, KH_2PO_4 and $CaCl_2$. **A** α-Amylase, **B** β-amylase, **C** dehydrogenase, **D** protease, **E** reducing sugars, **F** total soluble sugar and **G** protein. Four independent experiments were performed for each treatment designed condition, and different letters indicate significant differences among the averages ± SD, at $P < 0.05$ according to the Duncan test. Treatment conditions and preparation steps of seeds were as described in Fig. 1

and $CaCl_2$ (group 1 treatments) for 3, 6, 9 and 12 h or some chemicals including in group-2 (Fig. 1). Some preliminary experiments at different priming temperatures and times were also performed using ethanol, ascorbate, GA3, PEG, and Johnson solution (data not shown). Then the treatments were screened from the germination experiment based on FGP score, and the bests were applied as group-2 (Fig. 1).

Among the scratched seeds which were treated by the chemicals from group-2 treatments (Fig. 4), α-amylase, β-amylase, and dehydrogenase activities were reduced, and total sugar contents were increased by the following order;

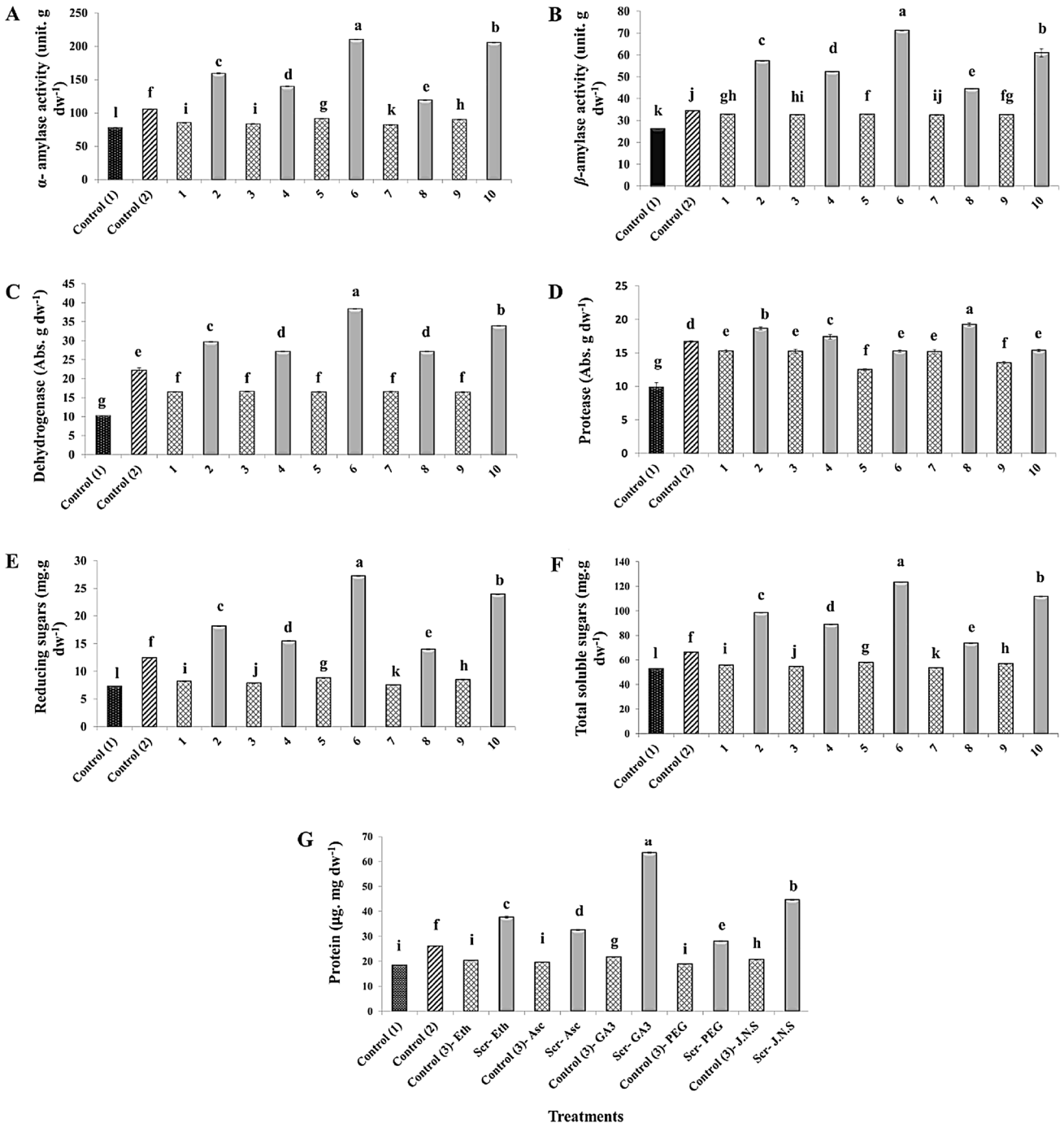

Fig. 4 Enzyme activities, total soluble sugar and protein content of *D. kotschyi* Boiss. seeds one day after priming with group-2 of treatments including; ethanol (Eth), ascorbate (Asc), gibberellic acid (GA3), polyethylene glycol (PEG) and Johnson nutrient solution (J.N.S). **A** α-Amylase, **B** β-amylase, **C** dehydrogenase, **D** protease, **E** reducing sugars, **F** total soluble sugar and **G** protein. Six independent experiments were performed for each treatment designed condition, and different letters indicate significant differences among averages ± SD, at $P < 0.05$ according to the Duncan test. Treatment conditions and preparation steps of seeds were as described in Fig. 1

Scr-GA3-Cold ≥ Scr-J.N.S > Scr-Ethanol > Scr-Asc ≥ Scr-PEG (Fig. 4A–C, E, F). These findings show the highest induction effect for Scr-GA3-Cold treatment on the activities of hydrolytic enzymes that have been mentioned about GA3 effects in other studies. The induction effect of the Johnson nutrient solution was also impressive (Fig. 4). To our

Table 1 Germination indices of primed and non-primed *D. kotschyi* seeds into Petri dishes by group-1 or group-2 treatments, during 15 days included germination start day (GSD), final germination percentage (FGP), mean germination time (MGT), time of reach to 50% of germination (T_{50}), germination rate index (GRI) and Timson index (TI)

Treatment	GSD	FGP (%)	MGT (day)	T50 (day)	GRI	TI (day^{-1}) %
(Group-1)						
Control (1), (3)	0.0d	0.0i	0.0i	0.0f	0.0l	0.0i
Control (2)	6.0b	34h	9.3d	9.0b	1.9k	2.1h
Scr-KNO_3 (3h)	5.0a	83a	8.2f	7.4d	5.5a	5.2a
Scr-KNO_3 (6h)	5.0a	75b	7.9g	7.6d	5.2b	4.7b
Scr-KNO_3 (9h)	5.0a	62d	6.9h	6.3e	4.7c	3.8d
Scr-KNO_3 (12h)	5.0a	56e	6.7h	6.0e	4.3d	3.5e
Scr-Na_2SiO_3 (3h)	6.0b	74b	9.7bc	9.3ab	4e	4.6b
Scr-Na_2SiO_3 (6h)	6.0b	62d	9.7bc	9.3ab	3.4f	3.9d
Scr-Na_2SiO_3 (9h)	6.0b	56e	9.6c	9.2ab	3.1g	3.5e
Scr-Na_2SiO_3 (12h)	7.0c	48f	9.8a-c	9.3ab	2.6ij	3.0f
Scr-KH_2PO_4 (3h)	6.0d	72b	9.7bc	9.2ab	3.9e	4.5b
Scr-KH_2PO_4 (6h)	6.0d	53e	9.9ab	9.6a	2.8h	3.3e
Scr-KH_2PO_4 (9h)	6.0b	45fg	9.7bc	9.3ab	2.4j	2.8fg
Scr-KH_2PO_4 (12h)	6.0b	36h	9.7bc	9.4a	1.9k	2.2h
Scr-$CaCl_2$ (3h)	7.0c	68c	10a	9.4a	3.5f	4.2c
Scr-$CaCl_2$ (6h)	7.0c	47fg	9.0e	8.5c	2.7hi	2.9fg
Scr-$CaCl_2$ (9h)	6.0b	43g	8.0g	7.5d	2.8hi	2.7g
Scr-$CaCl_2$ (12h)	6.0b	33.5h	8.0g	7.5d	2.1k	2.1h
(Group-2)						
Control (1)	0.0e	0.0f	0.0g	0.0f	0.0g	0.0f
Control (2)	5.0c	36d	8.6b	7.8b	2.3e	2.3d
Control (3)-Eth	14d	20e	14.5a	14a	0.7f	1.3e
Scr-Eth	3.0b	97.3ab	5.5d	5.1d	10.1c	6.1ab
Control (3)-Asc	14d	20e	14.5a	14a	0.7f	1.3e
Scr-Asc	3.0b	96b	5.7d	4.5d	10c	6.0b
Control (3)-GA3	14d	20e	14.5a	14a	0.7f	1.3e
Scr-GA3	2.0a	100a	4.5f	3.9e	13.3a	6.3a
Control (3)-PEG	14d	20e	14.5a	14a	0.7f	1.3e
Scr-PEG	3.0b	91.3c	7.7c	7.5c	6.8d	5.7c
Control (3)-J.N.S	14d	20e	14.5a	14a	0.7f	1.3e
Scr-J.N.S	2.0a	98ab	4.8e	3.7e	12.2b	6.1ab

Treatment conditions and preparation steps of seeds were as described in Fig. 1

The values presented are the mean ± SD of four (group 1) and six (group 2) independent experiments and different letters indicate significant differences among treatments in each index of each group, at $P < 0.05$ according to the Duncan test

knowledge, applying a complete nutrient solution including micro + macro-elements (e.g., Johnson medium) is a new approach in seed germination, growth, and grain nutrient enrichment. More inductions of enzyme activities could confirm the positive effects and the higher increments of carbohydrate/protein contents in Scr-J.N.S primed seeds (Fig. 4) than when a single mineral or substance was applied (group-1 in Fig. 3). Both treatments could be compared to their relative controls. The evaluation of hydrolytic enzymes at the post-stage of priming showed that 7% ethanol was able to increase hydrolytic enzymes' activity. Therefore, it could be ranked after the Johnson medium, although it caused a higher protease induction than the Johnson medium (Fig. 4D).

Chl *a*, Chl *b* and total Chl contents in seedling's leaves grown from the scratched and primed seeds by chemical treatments of group-2 were higher than those from non-scratched seeds and controls 1–3. However, from group-1, only Scr-(group-1)-3h caused an increase in Chl *a*, but Chl *b* was decreased inversely (Table 3). Total carotenoid and TCar/TChl increased by all Scr-(group-1)-3h, whereas TCar/TChl was decreased in Scr-Asc, Scr-GA3-Cold, and Scr-J.N.S, because of an increase in TChl content and a simultaneous decrease in TCar. The highest Chl *a*, Chl *b,* and TChl content of leaves was observed in the seedling grown from the scratched seeds treated by Scr-GA3-Cold (Table 3).

Table 2 Biomass and growth indices of 21-day-old *D. kotschyi* seedlings, grown into Petri dishes from primed seeds by group-1 or group-2 treatments

Treatment	SL (mm)	RL (mm)	SDW (mg)	RDW (mg)	Vig (I)	Vig (II)
(Group-1)						
Control (1), (3)	0.0q	0.0p	0.0p	0.0r	0i	0.0j
Control (2)	12.3h	15.0k	1.05o	0.28q	926.6h	45i
Scr-KNO_3 (3h)	13.1a	16.0a	1.30a	0.50a	2413.4a	149a
Scr-KNO_3 (6h)	12.6e	15.6d	1.26c-e	0.46e	2115.0b	129b
Scr-KNO_3 (9h)	12.2i	15.3h	1.22g-i	0.42i	1688.2d	101d
Scr-KNO_3 (12h)	11.8m	14.0l	1.18k-m	0.38m	1477.8e	87e
Scr-Na_2SiO_3 (3h)	12.9b	14.8b	1.29ab	0.49b	2135.1b	132b
Scr-Na_2SiO_3 (6h)	12.5f	15.5e	1.25d-f	0.45f	1736.0d	105d
Scr-Na_2SiO_3 (9h)	12.1j	15.2i	1.21h-j	0.41j	1527.6e	91e
Scr-Na_2SiO_3 (12h)	11.7n	14.7m	1.17l-n	0.37n	1269.8fg	74fg
Scr-KH_2PO_4 (3h)	12.8c	15.8c	1.28a-c	0.48c	2059.2b	127b
Scr-KH_2PO_4 (6h)	12.4g	15.4f	1.24e-g	0.44g	1462.2e	88e
Scr-KH_2PO_4 (9h)	12.0k	15.0j	1.20i-k	0.40k	1203.6fg	71fg
Scr-KH_2PO_4 (12h)	11.6o	14.6n	1.16mn	0.36o	930.1h	54h
Scr-$CaCl_2$ (3h)	12.7d	15.7d	1.27b-d	0.47d	1917.0c	118c
Scr-$CaCl_2$ (6h)	12.3h	15.3g	1.23f-h	0.43h	1284.6f	77f
Scr-$CaCl_2$ (9h)	11.9l	14.9k	1.19j-k	0.39l	1154.6g	68g
Scr-$CaCl_2$ (12h)	11.5p	14.5o	1.15n	0.35p	871.0h	50hi
(Group-2)						
Control (1)	0.0i	0.0h	0.0h	0.0f	0.0h	0.0h
Control (2)	12.2f	14.9f	1.1 f	0.28d	975.6f	48f
Control (3)-Eth	9.8h	12.0g	0.9g	0.24e	436.7g	24g
Scr-Eth	13.0c	16.0c	1.8c	0.34b	2822.7c	204c
Control (3)-Asc	9.8h	12.0g	0.9g	0.24e	436.7g	24g
Scr-Asc	12.8d	15.5d	1.7d	0.31c	2720.0d	193d
Control (3)-GA3	9.8gh	12.1g	0.9g	0.24e	43,767.7g	24g
Scr-GA3	14.0a	18.0a	3.0a	0.40a	3280.0a	340a
Control (3)-PEG	9.9g	12.1g	0.9g	0.24e	439.0g	24g
Scr-PEG	12.5e	15.4e	1.6e	0.27d	2546.7e	174e
Control (3)-J.N.S	9.8gh	12.0g	0.9g	0.24e	4373g	24g
Scr-J.N.S	13.4b	18.0b	2.8b	0.38a	3068.9b	310b

Shoot length (SL), root length (RL), shoot dry weight (SDW), root dry weight (RDW), vigor index (I) (Vig I), and vigor index (II) (Vig II). Treatment conditions and preparation steps of seeds were as described in Fig.1

The values presented are the mean ± SD of three independent experiments, and different letters indicate significant differences among treatments in each index of each group, at $P < 0.05$ according to the Duncan test

Seed priming treatments exert positive effects on seed germination

According to our preliminary tests (data not shown), control 1 started the first germination on the 21st day of seed soaking, but scarification could pull it off to 5–6th day (Table 1). Table 1 shows the germination indices after soaking of primed and non-primed seeds into Petri dishes. The earliest start day of germination (GSD) for the scratched seeds (control 2) was observed on 5th–6th day, whereas scratched treated seeds with Scr-GA3-Cold and Scr-J.N.S were able to bring GSD earlier to 2nd day (Table 1). Notably, Scr-Eth, Scr-Asc, and Scr-PEG treated seeds (scratched) also showed GSD on 3rd day (Table 1).

In the present study, FGP increased to 98–100%, respectively, for Scr-Eth and Scr-J.N.S on 8–9th day, and Scr-GA3-Cold on 7th day as the best results (Table 1, Fig. 5A). Nevertheless, a lower germination percentage (~ 80%) was obtained by Scr-KNO_3-(3h) treatment on 8th day (Fig. 5B). Priming of unscratched seeds by group-2 treatments (Eth, Asc, GA3, PEG, and J.N.S.), which was denoted as control 3, raised the germination about 20% with the first germination on 14th day (Table 1, Fig. 5A). In contrast, control 3 from group-1 (i.e., priming of unscratched seeds by each

Table 3 Pigment content indices of 21-day-old *D. kotschyi* seedlings, grown into Petri dishes from primed seeds by group-1 or group-2 treatments

Treatment	Chl *a* (µg mg FW^{-1})	Chl *b* (µg mg FW^{-1})	TChl (µg mg FW^{-1})	TCar (µg mg FW^{-1})	TCar/TChl
(Group-1)					
Control (1), (3)	0.0c	0.0c	0.0c	0.0c	0.0c
Control (2)	0.19b	0.12a	0.30a	0.04b	0.14b
Scr-KNO_3 (3h)	0.20a	0.08b	0.28b	0.06a	0.21a
Scr-KNO_3 (6h)	0.19b	0.12a	0.30a	0.04b	0.14b
Scr-KNO_3 (9h)	0.19b	0.11a	0.30a	0.05b	0.15b
Scr-KNO_3 (12h)	0.19b	0.12a	0.30a	0.04b	0.15b
Scr-Na_2SiO_3 (3h)	0.20a	0.08b	0.28b	0.06a	0.21a
Scr-Na_2SiO_3 (6h)	0.19b	0.12a	0.30a	0.05b	0.15b
Scr-Na_2SiO_3 (9h)	0.19b	0.12a	0.30a	0.05b	0.15b
Scr-Na_2SiO_3 (12h)	0.19b	0.11a	0.30a	0.05b	0.15b
Scr-KH_2PO_4 (3h)	0.20a	0.08b	0.28b	0.06a	0.21a
Scr-KH_2PO_4 (6h)	0.19b	0.12a	0.30a	0.04b	0.15b
Scr-KH_2PO_4 (9h)	0.19b	0.12a	0.30a	0.04b	0.14b
Scr-KH_2PO_4 (12h)	0.19b	0.11a	0.30a	0.05b	0.15b
Scr-$CaCl_2$ (3h)	0.20a	0.08b	0.28b	0.06a	0.21a
Scr-$CaCl_2$ (6h)	0.19b	0.11a	0.30a	0.05b	0.16b
Scr-$CaCl_2$ (9h)	0.19b	0.11a	0.30a	0.05b	0.15b
Scr-$CaCl_2$ (12h)	0.19b	0.11a	0.30a	0.05b	0.15b
(Group-2)					
Control (1)	0.0f	0.0f	0.0g	0.0d	0.0e
Control (2)	0.22d	0.09e	0.31e	0.07a	0.23a
Control (3)-Eth	0.21e	0.09e	0.30f	0.03c	0.10c
Scr-Eth	0.24b	0.20c	0.44c	0.06a	0.14b
Control (3)-Asc	0.21e	0.09e	0.30f	0.05b	0.16b
Scr-Asc	0.24b	0.20c	0.44c	0.03b	0.07cd
Control (3)-GA3	0.18e	0.09e	0.27f	0.06a	0.22a
Scr-GA3	0.25a	0.22a	0.47a	0.01c	0.02d
Control (3)-PEG	0.21e	0.09e	0.30f	0.06a	0.20a
Scr-PEG	0.23c	0.13d	0.36d	0.06a	0.17b
Control (3)-J.N.S	0.21e	0.09e	0.30f	0.06a	0.20a
Scr-J.N.S	0.24b	0.21a	0.45b	0.02c	0.04d

Indices included; chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophyll (TChl), total carotenoid (TCar), and total carotenoid/chlorophyll (TCar/Chl). Treatment conditions and preparation steps of seeds were as described in Fig.1

The values presented are the mean ± SD of four (group 1) and six (group 2) independent experiments, and different letters indicate significant differences among treatments in each index of each group, at $P < 0.05$ according to the Duncan test

KNO_3, Na_2SiO_3, KH_2PO_4, and $CaCl_2$) caused no germination (Table 1), suggesting a more positive and inductive effect of the group-2 treatments on seed germination than group-1, even when the seeds have not been scratched. The enzyme activities of control 3 from group-2, which were often slightly higher than those in control 1, can confirm this claim (Fig. 4). Another confirmation could be obtained via priming of seeds by joint scratch + chemical treatments of group-2 (Fig. 4 and Table 1), which caused more significant positive influences on seed germination indices than the same work by group-1 (Fig. 3 and Table 1). Morphologic differences among the 15-d-old grown seedlings could be observed in Figs. 6 and 7. The highest lengths and dry weights of shoots, roots, as well as Vig (I) and Vig (II), were observed at seedlings treated firstly by Scr-GA3-Cold and secondly Scr-KNO_3-3h (Table 2).

In addition to GA3 and KNO_3, most of the other chemical treatments from group-1 (especially, 3 and 6 h), and also all chemical treatments from group-2 when applied on scratched seeds, caused an increase in the SL and RL of seedlings (Table 2), which indicates the advantages of priming technique. However, 12 h treatment by group-1 caused the lowest

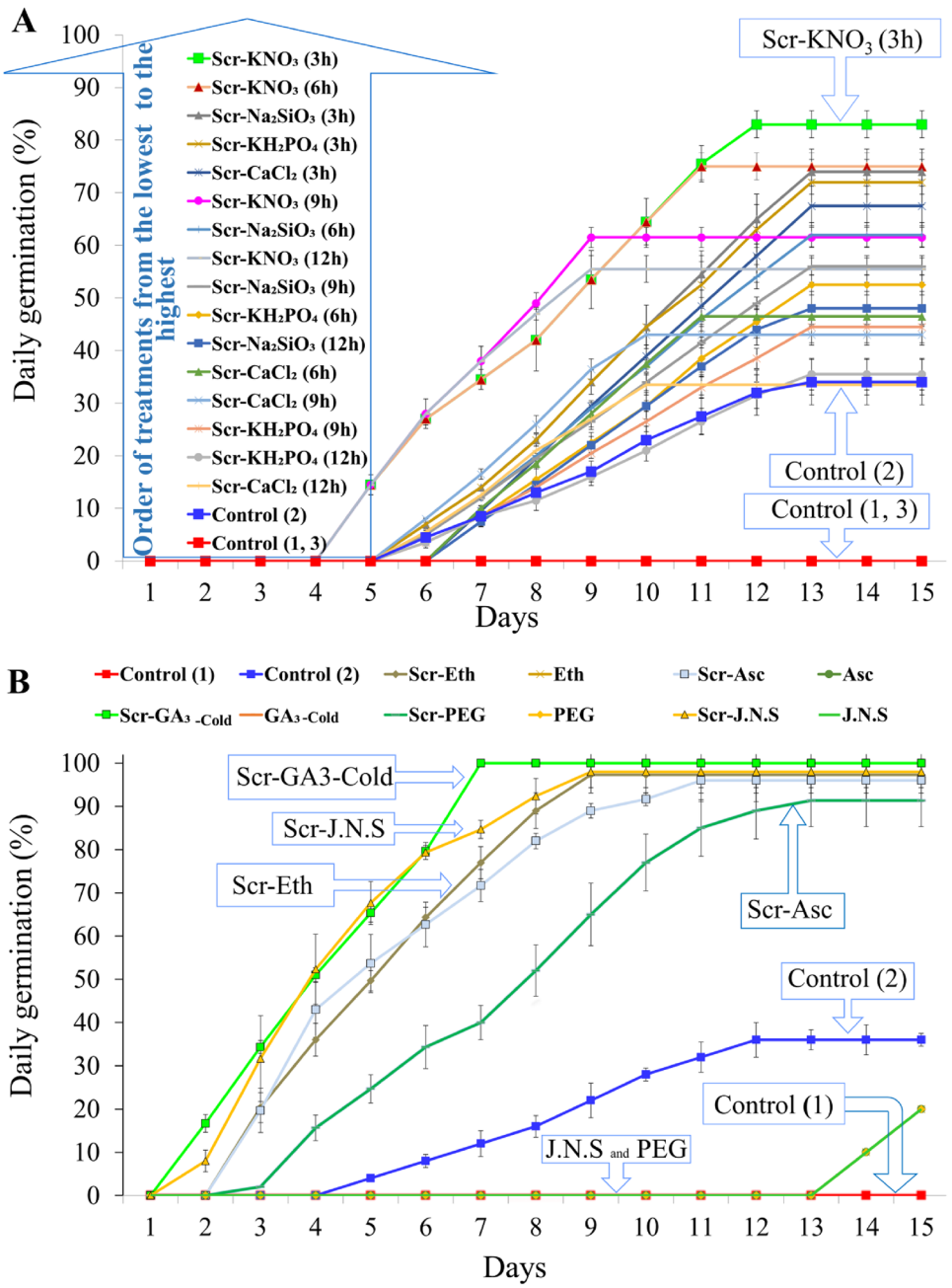

Fig. 5 Daily FGP of *D. kotschyi* Boiss., during 15 days after seed priming with group-1 of treatments including; KNO_3, Na_2SiO_3, KH_2PO_4 and $CaCl_2$, and group-2 of treatments including; ethanol (Eth), ascorbate (Asc), gibberellic acid (GA3), polyethylene glycol (PEG) and Johnson nutrient solution (J.N.S). The values presented are the mean ± SD of four (for group-1) and six (for group-2) independent experiments. Treatment conditions and preparation steps of seeds were as described in Fig. 1

RL (Table 2). The results showed that the growth indices of seedlings were almost more improved by group-2 than by group-1 seed treatments, in comparison to their relative controls (Table 2). On the other hand, the highest positive effect of group-2 treatments was related to the scratched treated seeds, and non-scratched seeds resulted in the seedlings with lower growth (Table 2).

Discussion

The influence of mechanical scarification on hydrolytic enzyme activities and metabolite levels could indicate some problems related to the seed coat permeability of *D. kotschyi*. Therefore, after removing this barrier, the

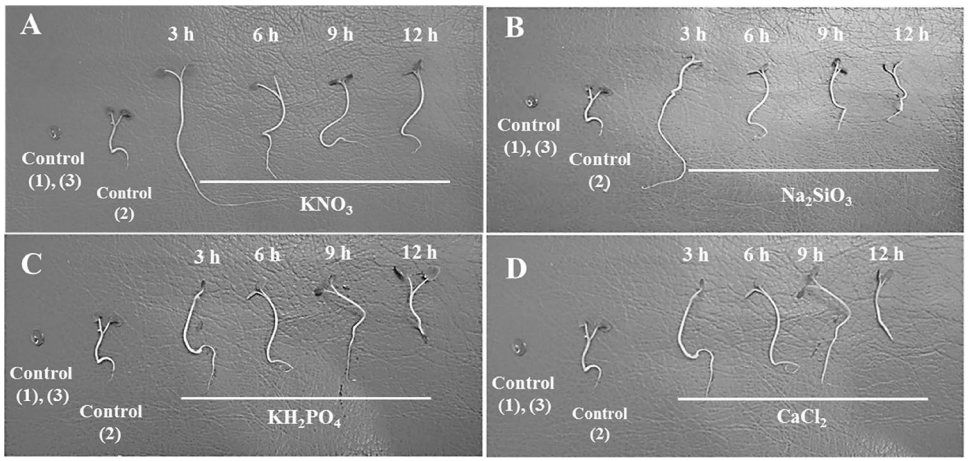

Fig. 6 Fifteen-day-old seedlings of *D. Kotschyi* Boiss. grown from primed seeds by group-1 (**A**–**D**). Treatment conditions and preparation steps of seeds were as described in Fig. 1

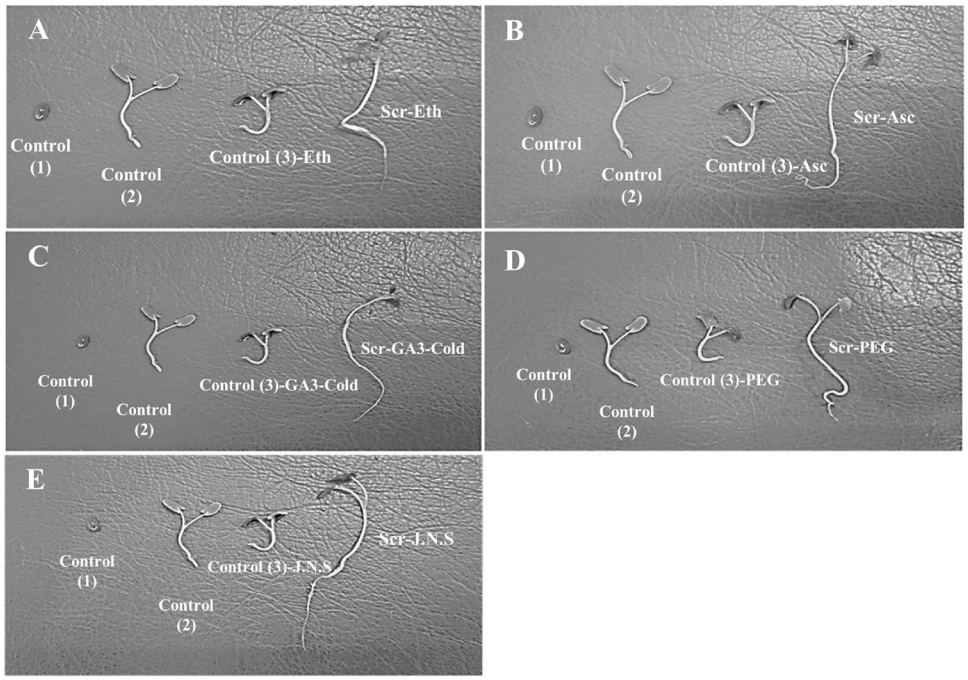

Fig. 7 Fifteen-day-old seedlings of *D. Kotschyi* Boiss. grown from primed seeds by group-2 (**A**–**E**). Treatment conditions and preparation steps of seeds were as described in Fig. 1

increases in physiological and biochemical processes would not be unexpected. Mechanical scarification by sandpaper has already been used to overcome the hard seed coat of *D. kotschyi* Boiss (Fattahi et al. 2011) and *D. argunense* Fischer ex Link (Chang et al. 2009). However, the details of the method were not reported, especially in *D. kotschyi*.

Effects on hydrolytic enzymes and metabolite levels

According to the literature, α-amylase and β-amylase are de novo synthesized during the priming to help reserve utilization for respiration and biosynthesis of macromolecules (De Lespinay et al., 2010). The activity of α-amylase has also been introduced as an efficient seed dormancy level marker (Vieira et al., 2002). Dehydrogenases are essential enzymes during seed imbibition. A burst in dehydrogenase activity after seed imbibition and before radicle emergence was observed in cowpeas (Oaikhena et al. 2013). In the current experiment, the highest enzyme activities were often observed at lower priming times, e.g., 3 or 6 h (for all treatments of group-1), and the enzyme activities were decreased by increasing the priming time to 12 h (Fig. 3A–C). The same pattern was observed for the content of reducing sugars but not entirely for total soluble sugar content (Fig. 3E, F). As a possible reason, some other hydrolytic enzymes (e.g., beta-glucanase, xylanase, glucoamylase, and alfa-glucosidase), which have not been evaluated in this research, might be affected differently by the various used priming times and caused the difference in total soluble sugar content

(Kossmann and Lloyd 2000; Muralikrishna and Nirmala 2005; Joshi 2018). In another study, the increased activity of hydrolytic enzymes (e.g., amylases, protease, lipase, etc.) in *Cicer arietinum* L. soaked seeds showed the seed storage loosening and degradation of reserve nutrients which consequently led to accelerated seed germination and seedlings growth (Rahman et al. 2008). Investigations revealed that dehydrogenases in the anaerobic phase of seed germination could catalyze the stored products. The anaerobic respiration occurs during the resting stages of seed life and the primary steps of seed germination (Jones et al. 1999).

Proteolytic enzymes have fundamental roles in the biochemical mechanisms of seed germination and protein degradation (Padmakar et al. 2005). Protein levels in sprouting seeds result from two contrasting pathways; specific protein synthesis involving reserves mobilization, which led to the embryo germination, and inversely, destruction of storage proteins due to the activities of proteases (Rajeswari and Ramakrishna Rao 2002; Wang et al. 2007). A part of the increased protein level resulting from most treatments is attributed to de novo synthesis of amylases (Lovegrove and Hooley 2000). In this study, the synthesis or degradation of proteins might be influenced by the priming treatments and/or priming times. Other investigations also showed that priming improved photosynthetic pigment amounts (Chl *a*, Chl *b,* and carotenoids) and enhanced seedling vigor and performance. The improvement of photosynthetic pigments points to seed priming's role in positively influencing Chl and carotenoid synthesis in the seedlings (Jamil et al. 2013; Jisha and Puthur 2014).

In the current study, enzymes and metabolite evaluations in primed seeds showed that scarification had a key role in removing dormancy and activation of metabolism and that the addition of chemical treatments, such as ethanol or ascorbate, especially GA3-Cold, was able to add further advantages over scarification alone (Figs. 3 and 4). The reverse results were stated by Vargas-Simón et al. (2017), who reported no effects by the addition of GA to *Ormosia macrocalyx* Ducker scratched seeds. This may suggest the existence of some internal and physiological reasons and the external ones related to the seed coat, in seed dormancy of *D. kotschyi*.

The application of micro/macro-elements has recently been reported in seed priming and seed coating studies (Muhammad et al. 2015; Sayadiazar et al. 2016). Generally, the researchers believe that these methods possibly are easy and cost-effective alternatives for resource-poor farmers, especially in developing countries. The main reason is that the prevalent practices of chemical fertilizers added to the soil or using them as foliar applications need high quantities and quality of micronutrient fertilizers. Still, seed priming with small amounts of a complete nutrient solution seems to be more economical (Farooq et al. 2012). Ethanol has been mentioned to break down the dormancy and stimulate the germination of seeds in rare previous research (Bewley and Black 2012; Afzal et al. 2013). The recent researchers suggested a relationship between ethanol's positive effects (2% and 4% ethanol levels) on tomato seed germination with its influence on the induction of antioxidant enzymes. They also reported that priming with a higher ethanol concentration (6%) was unsuccessful in improving seed germination and seedling growth because of decreased antioxidative activity. In the present study, 7% ethanol increased hydrolytic enzymes activity at the post-stage of priming.

Seed priming ameliorated seed germination

Chemical priming has been used to augment seed germination and seedling establishment in many previous investigations (Shafiq et al. 2015; Hussein 2016; Saddiq et al. 2019). It has been reported that when soaking was accompanied by subsequent desiccation, in some cases priming has not showed positive effects. Because the benefits to germination might be lost during drying, or in some cases, remained on desiccation but disappeared fast in storage (Heydecker and Gibbins 1978). The current study showed that the enzyme activities significantly increased in primed seeds, and the germination indices were also highly improved in primed seeds after seed sowing (Figs. 3, 4, and 5; Tables 1). These results suggest that drying (at least at the desiccation stage in *D. kotschyi* seeds) could not destroy a 'priming memory' produced during the priming process (Chen and Arora 2013).

In the present study, FGP increased to 98–100%, respectively, for Scr-Eth and Scr-J.N.S on 8–9th day and Scr-GA3-Cold on 7th day (Table 1, Fig. 5B). However, a lower germination percentage (~ 80%) was obtained by Scr-KNO_3-(3 h) treatment on 8th day (Fig. 5A). In comparison, the highest FGP 60–70%, was obtained by the sandpaper or 10 min in sulfuric acid on 15th, and an FGP of 80% was gained by 50 ppm GA3 on 30th day, in Fattahi and his co-worker's experiments (2011). The reasons for the differences possibly are related to the type of treatments, the higher used concentration of GA3 in these experiments (500 ppm), and different seed germination conditions. The pinpoints are the positive effects of most of the treatments applied in this study, particularly group-2 treatments (Figs. 3, 4, 5, 6, 7; Tables 1, 2).

Although they did not mention how the sandpaper treatment was applied, they could improve the GSD from 21st day to 7th day. They also reported FGP 60% on the 15th day of their experiments by sandpaper, which was about 34–36% on the same day of this investigation. The earlier start time of germination in this research and lower FGP, may be related to the difference in two growth conditions or substrates/media (perlite pots *vs*. Petri dishes) and the various sandpaper application methods as well. Other researchers reported

nearly 58% germination on 14th day for *D. argunense,* by 50 times rubbing the seeds with the pieces of sandpaper (Chang et al. 2009), whereas it was done only by 4 times dragging over 10 cm long sandpaper in our experiments.

The highest FGP (60%) was obtained by Chang et al., (2009) by pre-soaking of *D. arunense* Fischer ex Link, for 30 s in sulfuric acid. In another study, 63–85% was gained by treating scratched seeds of *D. parviflorum* Nutt. in hot water (100 °C) (Van Veldhuizen and Knight 2006). However, 500 and even 1000 ppm GA3 in the latest research caused only 25–26% FGP compared to 10% FGP in control at the end of 21 days (Van Veldhuizen and Knight, 2006), and sulfuric acid caused only a 0–2 FGP. These data show that a dependency on the type of studied species might also be involved in *Dracocephalum* seeds response. The least MGT and T50 (~4–5 days) and the highest GRI (~13.3) and TI (~6.3) were also related to first Scr-GA3-Cold, and then Scr-J.N.S, Scr-Eth, Scr-Asc, and in some cases to the KNO_3 treated seeds (Table 1). The comparable amounts for MGT, T50, and GRI in Fattahi et al. (2011) were, respectively, 13.07, 10, and 1.76. Previous studies showed that priming of seeds with GA3 and KNO_3 could improve the germination of tomato seeds and reduce mean germination time (Bocian and Holubowicz 2008; Tzortzakis 2009).

According to the literature, in addition to the effect of GA3 on amylase production, this hormone could affect cell elongation by increasing cell wall expansion and causing water to enter into cells (Arteca 1996). However, the highest mean root DW (~0.5 mg) was observed in seedlings grown from Scr-KNO_3-3h primed seeds. The highest mean shoot DW (~3 mg) belonged to seedlings grown from the primed seeds with Scr-GA3-Cold. The increases in SL, RL, and dry matter production could be due to the earlier start of emergence and were in agreement with the previous studies on maize (Afzal et al. 2008). Physiological dormancy occur when the germination and growth-promoting enzymes or hormones are inhibited by some inhibitors such as ABA, leads to germination prevention. These inhibitors, at a adequate level, neutralize influences of germination-promoting enzymes, such as gibberellins. Normally, the balance between inhibitors and promoters should be in favor of those that will let germination to progress (Mousavi et al. 2011). Overall, Scr-GA3-Cold showed the best positive influences in this research. The positive effects of gibberellin treatment, which have been mentioned in other studies, are included; breaking down the seed dormancy, promoting germination, inducing cell divisions and increasing inter-node length in plants (Rood et al. 1990; Naeem and Muhammad 2006; Ghodrat and Rousta 2012).

Conclusion

According to some previous works, to break the seed dormancy of *D. kotschyi* Boiss., the best results were obtained by seed pre-soaking in sulfuric acid for 15 min or 0.2 ppm BAP or in 15 ppm GA3, which were resulted in an FGP 86–90% on 15th day of seed culture. Other researchers have got 60–70% germination on 15th day by applying sandpaper or 10 min treatment in sulfuric acid. In this research, seed priming as the latest technology in engineered seed nutrition and a useful advance in modern farming practice was used instead of seed pre-soaking. Our results showed that 98–100% germination is accessible on the 2–3th day by combining mechanical scratching together with each of GA3-Cold/nutrient solution/ethanol/ascorbate treatments. This research also demonstrated that scarification alone is much more effective than chemical treatments on *D. kotschyi* seed germination, indicating the seed coat induced-dormancy is a stronger inhibition factor than embryo dormancy in *D. kotschyi*. However, the joint priming treatment (scarification + chemical) was much more effective than mechanical scrification. This suggests that seed dormancy in *D. kotschyi* could be related to both external (physical) and internal (physiological–enzymatic) factors, with the priority of physical reasons. The applied treatments could be sorted by the following order based on their positive effects on seed germination and seedling growth; Scr-GA3-Cold ≥ Scr-J.N.S > Scr-Eth ≥ Scr-Asc > Scr-PEG > Scr-KNO_3-3h > Scr-other treatments > Scarification alone (Control 2) > chemical-priming alone (Control 3).

Priming can increase the production and propagation of endangered plants' dormant seeds, especially the medicinal ones, by accelerating germination and uniformity. This can be done by farmers or in research centers, allowing the survival of these plants and the possibility of their redistribution in pastures, as well as the collection of abundant biomass of the plant to utilize their medicinal properties.

Author contribution statement MMH designed, and RG performed the experiments; MMH, RG and MH analyzed the data; RG made a preliminary draft; MMH, AR and MH wrote the manuscript. MMH, AR and MH constructed and improved the figures. MH reviewed and edited the manuscripts. All authors have read and approved the final version of the manuscript.

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Declarations

Conflict of interest There is no conflict of interest. Authors declare that using of endangered medicinal plant (*Dracocephalum kotschyi* Boiss.) is only for research aims and not for business/commercial goals, which was informed and was with the permission of the research council of University.

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