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Dormancy removal in pistachio nut: Influences of Hydrogen Cyanamid (Dormex®) as compared to ordinary seed chemical pre-treatments

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Abstract: Seed germination is considered an important phenological stage of plant growth during which the viable, non-dormant embryos develop into seedlings under favorable conditions. Currently, various seed priming treatments may be used to enhance germination values (germination percentage, Lag phase, time to reach 50% germination) and breaking dormancy; gibberellins and KNO_3 are applied as common treatments in this regard. In the present study, efficacy of Hydrogen Cyanamid (HCN, Dormex®) on seed germination of two cultivated pistachio varieties (Abasali, Shahpasand) were studied and the results were compared to ordinary ingredients, namely gibberellic acid (GA_3) and KNO_3. The extraction of parameters was carried out using four-parameter Hill function. The results reveal that HCN may significantly improve critical parameters involved in pistachio seed germination. In Shahpasand variety, seeds treated with HCN germinated prior to other treatments (150.53 hours after treatment) and achieved 50% germination earlier than other treatments (199.00 hours only). According to the results, utilization of HCN in seed testing experiments may be encouraged.

1. Introduction

In agriculture, seeds are considered as starting points of plant growth. Assuming seeds are non-dormant, germination is the key component of seedling emergence (Forcella *et al.,* 2000). Germination may be defined as the process associated with the initiation and completion of embryo emergence; it refers to the progress of a seed from imbibition through radicle emergence (Gniazdowska *et al.,* 2010). Germination parameters are useful for estimating the conversion of seeds to seedlings and, thus, the suitability of a seed lot for commercial seedling production. Germination parameters are also useful in determining the type of seed pre-treatment as well as nursery management practices needed to attain a high level of germination (Kolotelo *et al.,* 2001). Abscisic acid (ABA) induces dormancy during maturation, and gibberellins (GAs) play a key role in dormancy release and in the promotion of germination (Kaur *et al.,* 2006). In many cases, endogenous levels of these hormones (GA, ABA) are associated with the ability of the seeds to germinate (Seo *et al.,* 2006); seed dormancy must be broken to induce germination. In morphophysiological dormancy, seeds must be exposed to cold, heat, gibberellic acid, or chemical materials for dormancy breaking (Hilton, 1984; Dehghanpour *et al*., 2011). The use of potassium nitrate has been an important seed treatment in seed-testing laboratories for many years without a good explanation for its action mechanism (Çetinbaş and Koyuncu, 2006). Hydrogen cyanamide (HCN Dormex®) has been identified as one of the most effective dormancy-breaking agents in many deciduous plant species (Siller-Cepeda *et al.,* 1992) and has been exploited mainly as an artificial rest-breaking agent to stimulate budbreak in mild winter climates (Ben Mohamed *et al.,* 2012). However, the emission of HCN has been detected during the pre-germination period of many seeds (Gniazdowska *et al,*. 2010).

Pistachio (*Pistacia vera* L.), a member of Anacardiaceae family (Khan *et al.,* 1999), is a dioecious species and for this reason it has acquired large genetic diversity (Behboodi, 2002). Seed dormancy, oily nature of cotyledons leading to early spoilage, risk of heterozygosis, and production of male/female plantlets are major problems associated with pistachio sexual propagation (Jangali Baygi, 2012). Therefore, under natural conditions, an erratic and irregular germination over a quite long period. On the other hand, Pistachio plants cannot be readily propagated from cuttings taken from mature trees and are currently propagated by grafting on seedling rootstocks (Behboodi, 2002). Further-

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more breeding programs and development of hybrid seedlings/superior rootstocks require a rapid and reliable method for seed germination (Ghazaeian *et al*., 2012). Hence, in the present study, effects of HCN on dormancy removal and seed germination of two pistachio varieties were studied and the results were compared to widespread chemical treatments such as GA_3 and KNO_3 as well as cold stratification.

2. Materials and Methods

The present study was undertaken in the physiology laboratory at the Horticulture Department, Gorgan University of Agricultural Sciences and Natural Resources, Golestan, Gorgan, Iran. Two Iranian cultivated pistachio varieties, ('Abbasali' and 'Shahpasand') were procured from a commercial orchard located in Damghan, Semnan Province.

Initially, the healthy and bold seeds were selected and the extracted kernels were treated for 24 h with different chemicals, *i.e.* GA_3 (50, 100 mg/l), KNO_3 (1.0, 1.5 %), and Dormex® (50, 100 mg/l), all procured from Sigma. Dormex is a mineral component with chemical formula H_2CN_2 (Siller-cepeda *et al.,* 1992). The chemical component utilized in the present study was aqueous Hydrogen Cyanamid (H_2CN_2, 187364 Sigma, F.W.= 42.04 g/mole). Initially the required amount of HCN was dissolved in a few drops of ethanol to prepare the stock solution from which the concentrations of 50-100 mg/l HCN were prepared with distilled water. The control seeds were also soaked in distilled water only and some seeds of the same lot were kept in a refrigerator (5°C) as cold stratification treatment. In order to prevent fungal contamination, the treated kernels were surface sterilized with sodium hypochlorite 10% and then rinsed at least three times with autoclaved water. These were then cultured in plastic containers filled with autoclaved sand (diameter 2 mm). The samples were observed daily for a period of 54 days and the number of germinated seeds was recorded. Radicle emergence (5 mm in length) was taken as germinated seed. The cumulative germination count of each pistachio variety seed lot was fitted to a four-parameter Hill function (4-PHF; Equation 1) already reported by El-Kassaby *et al.* (2008) as a mathematical representation and parameter extraction of seed germination:

$$(1) \qquad y = y_0 + \frac{ax^b}{c^b + x^b}$$

In the equation 1, y is considered as the percentage of cumulative germination at time *x*, y_0 is the intercept on the *y* axis (≤0), *a* is the asymptote, or maximum cumulative germination percentage, which is equivalent to germination capacity, *b* is a mathematical parameter controlling the shape and steepness of the germination curve and *c* is the "half-maximal activation level" measured in days and represents the time required for 50% of viable seeds to germinate ($G_{50\%}$). Following extraction of a, b, c and Y_0, from 4-PHF, the time at germination onset (lag) and the final germination percentage (G_{max}) were also computed using the following equations:

$$(2) \qquad \text{lag} = b\sqrt{\frac{-y_0 \; c^b}{a + y_0}}$$

$$(3) \qquad G_{max} = y_0 + a$$

The entire research work was designed as a completely randomized design (CRD) with factorial arrangement including seven treatments and three replications. The data were analyzed using SAS software.

3. Results and Discussion

Pistachio plants cannot be readily propagated from cuttings procured from mature trees and are currently propagated through budding/grafting on seedling rootstocks. Though tissue culture techniques were proposed as new and advanced methods for multiplication of this species (Behboodi, 2002) seedling development followed by budding is still considered a superior method for pistachio orchard establishment. However, seed dormancy and erratic germination cause significant trouble for growers, nurserymen, and fruit breeders (Jangali Baygi, 2012). Dormancy release is accomplished by diverse mechanisms that include complex interactions with the environment mediated by plant hormones and other small molecules, and is supposed to select conditions for germination that are most favorable for plant survival (Finkelstein *et al*., 2008). Application of GA_3. Thiourea, and KNO_3 as common priming agents were formerly reported in dormancy removal of some nut species such as wild pistachio (Khan *et al*., 1999), walnut (Kaur *et al.,* 2006), and pecan (Ghazaeian *et al*., 2012). Hence, the present study was also undertaken on two cultivated pistachio varieties and the following results were found.

The results of analysis of variance of the effects of chemical treatments on dormancy removal and germination parameters of pistachio varieties are shown in Table 1. The data demonstrate a sharp and considerable effect of chemical priming on germination percentage, the time at germination onset (lag), and time taken to 50% germination in each seed lot. Furthermore, the results demonstrate the effectiveness of Dormex® to release pistachio seeds from dormancy.

The lag phase is an important parameter to determine quality of seed lots. In Abbasali variety the control seeds germinated 731.28 h after culture, while the chemical priming considerably reduced this period. For example, seeds treated with 1.5% KNO_3 germinated at 263.26 h only. As shown in figure 1, application of Dormex® at the rate of 100 mg/l had the similar effect on lag phase and the germination at onset was recorded at 385 h following treatment. In the case of Shahpasand variety, Dormex® was found to be the best treatment to remove seed dormancy and the lag period from 150.35 h in control seeds was reduced to 52.78 h only. This result indicates that the priming treatment with Dormex® may lead to precocious and early seed

Table 1 - The results of analysis of variance of the effects of chemical treatments on dormancy removal and germination parameters of two cultivated pistachio varieties

Sorces of variations	d.f.	Mean square		
		D_{50}	G_{max}	lag
Variety	1	2508961.5**	1138.38**	1328137.5**
Treatments	6	66477.16**	911.36**	49107.3**
Variety x treatment	6	33053.44*	528.47**	21142.09*
Error	28	12569.26	87.34	17639.87
cv		19.68	12.28	52.83

$G_{max=}$ germination percentage.
D_{50}= time to reach 50% germination.
Lag= time to germination onset.

germination. An application of calcium cyanamide was previously proposed to overcome a lack of winter chilling in many plants (Walton *et al.,* 1991). Ben-Mohamed *et al.*, (2012) investigated the effect of HCN on dormancy release and carbohydrate metabolism in the buds of grapevine.

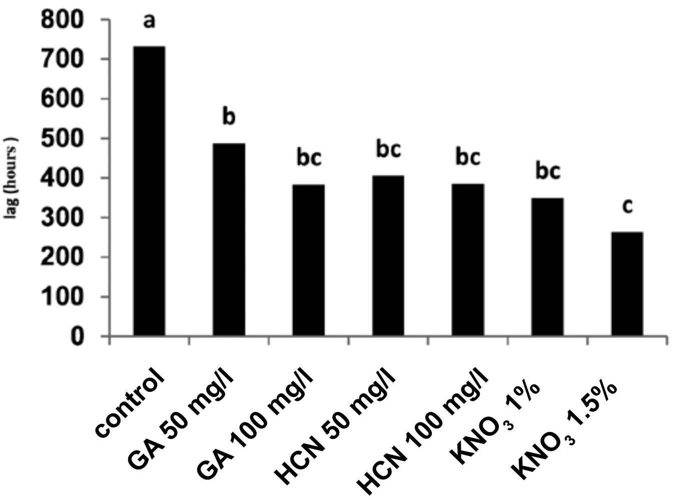

Fig. 1 - Effect of different priming agents on time to germination at onset (lag phase) of Abbasali pistachio variety.

Their results showed that application of HCN to dormant grape buds advanced budbreak to 10 days after treatment while the untreated buds opened within 14 days after treatment. Furthermore, HCN caused significant differences in the rate and percentage of budbreak (85% as compared to 42% budbreak in the controls). However, there are quite a few reports on the effect of this compound on seed dormancy. Gniazdowska *et al.* (2010) studied the role of HCN on breaking dormancy of apple embryos. Interestingly, they found that the deep dormancy of apple embryos may be removed by a quite short pre-treatment (3-6 h) of HCN. Furthermore, the development of abnormal seedlings was also lower than control, non treated seeds. Such effect of HCN in apple embryos is comparable with the response of Shahpasand pistachio variety in the present study (Fig. 2).

The time taken to reach 50% seed germination is also considered as a remarkable parameter in seed germination experiments. According to figures 3 and 4, application of chemical treatments significantly reduced this

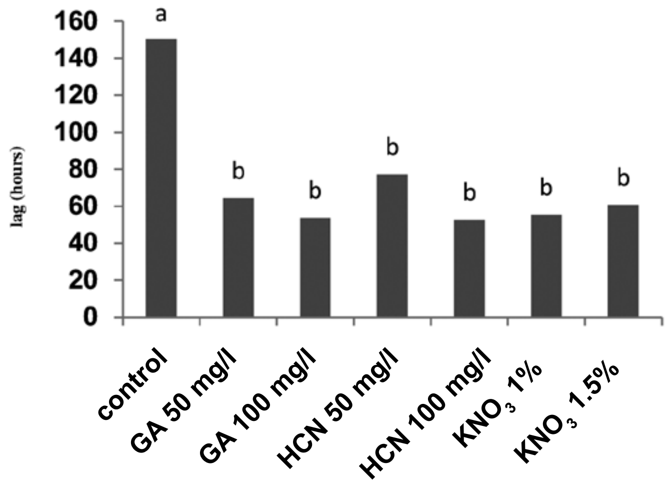

Fig. 2 - Effect of different priming agents on time to germination at onset (lag phase) of Shahpasand pistachio variety.

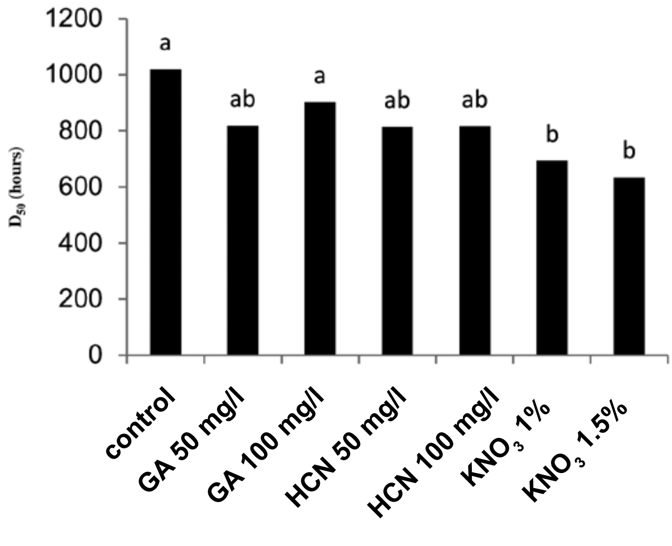

Fig. 3 - Effect of different priming agents on time to reach 50% germination ($G_{50\%}$) of Abbasali pistachio variety.

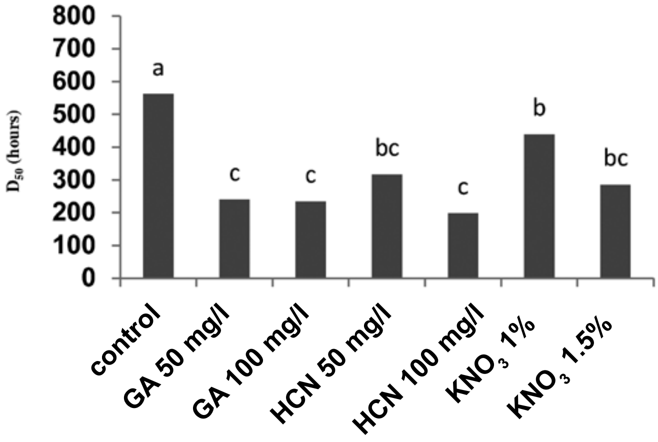

Fig. 4 - Effect of different priming agents on time to reach 50% germination ($G_{50\%}$) of Shapasand pistachio variety.

period. In Shahpasnd variety, the time taken to $G_{50\%}$ in control seeds was recorded as 562 h while in the case of priming treatments it was effectively reduced to 199 h (Dormex® 100 mg/l), 234 h (GA_3 100 mg/l) and 286 h (KNO_3 1.5%). In the case of Abbasali, the same trend was observed and KNO_3 1.5% was found to be the most effective treatment.

In both varieties, cumulative germination in control seeds was low but it was enhanced following chemical priming (Fig. 5 and 6). This means that G_{max} in 'Abbasali' increased from 46.23% in control seeds to 86.30% in seeds treated with KNO_3. The same variety had 81.91% of G_{max} when treated with Dormex®. Application of KNO_3 in 'Shahpasand' seeds led to 99.25% germination. Hence, the results clearly reveal that Dormex® was effective in dormancy removal and germination of pistachio nuts and that in some cases it was comparable or even better than GA_3 and KNO_3 treatments.

The effectiveness of gibberellins in seed germination has been reported in numerous studies (Bewley and Black, 1994; Seo *et al.*, 2006). *In vitro* germination of walnut (*Juglans regia* L.) embryos was undertaken by Kaur *et al.* (2006) and they used components such as GA_3, Benzyl adenine, and kinetin to improve embryo growth and development. They reported GA_3, along with low temperature, as the best treatment for walnut *in vitro* embryo germination. In another experiment for dormancy breaking of tulip seeds, Rouhi *et al.* (2010) also applied GA_3 at rates from 250 to 500 mg/l. They observed an enhancement of seed germination as GA_3 levels were increased. In general, two main functions have been proposed for gibberellins during seed germination process. First, GA increases the growth potential of the embryo. Secondly, it is necessary to overcome the mechanical restraint conferred by the seed-covering layers, by weakening the tissues surrounding the radicle (Kucera *et al*., 2005). Gibberellins stimulate germination by inducing hydrolytic enzymes that weaken the barrier tissues such as the endosperm or seed coat, inducing mobilization of seed storage reserves, and stimulating expansion of the embryo (Finkelstein *et al.,* 2008).

Potassium nitrate is considered to be one of the most effective and widely used components with respect to breaking seed dormancy (Çetinbaş and Koyuncu, 2006). As far as pistachio seed germination is concerned, Khan *et al.* (1999) applied KNO_3 at the rate of 1% to wild pistachio and they observed 26% seed germination as compared to control with only 2%. In an experiment carried out on *Prunus avium* L., the stratified seeds were treated with GA_3, KNO_3 and thiourea (Çetinbaş and Koyuncu, 2006). Treatments with 7,500 mg/l KNO_3 after 120 days of stratification were more effective, yielding 64.54% germination of seeds with coat. However, in case of seeds without coat (kernels), GA_3 was found to be more effective than other chemicals.

The role of HCN in dormancy removal may be attributed to its stimulatory effects on endogenous ethylene production. Ethylene increases respiration as well as starch degradation of seed reserves and the embryo then starts germination. A similar result was also reported for tobacco in which ethylene was involved in endosperm rupture and

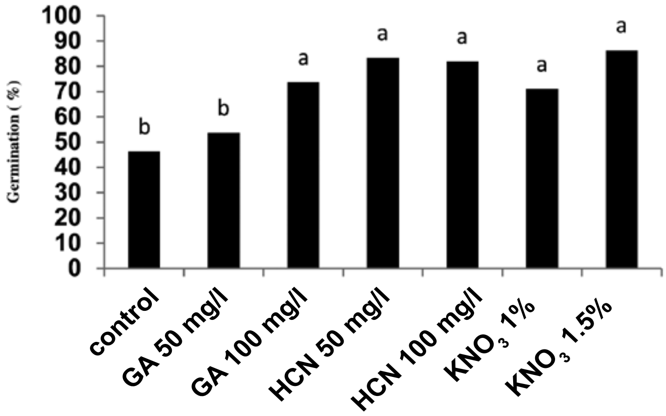

Fig. 5 - Effect of different priming agents on cumulative germination percentage (G_{max}) of Abbasali pistachio variety.

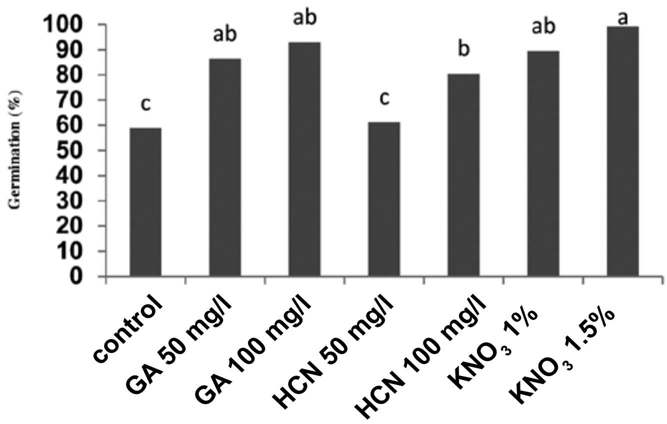

Fig. 6 - Effect of different priming agents on cumulative germination percentage (G_{max}) of Shahpasand pistachio variety.

high bGlu I expression during seed germination (Kucera *et al*., 2005). Ben Mohamed *et al*. (2012) also stated that starch concentration was high in dormant buds of grapevine and declined rapidly to reach its lowest level following HCN application. The same process may occur in treated pistachio seeds leading to 99.25% germination in Shahpasand variety (Fig. 6). Another reason for the role of HCN in improvement of seed germination can be the activation of the pentose-phosphate pathway which is thought to be necessary for the breaking of dormancy in buds and seeds (Walton *et al*., 1991). They demonstrated that application of HCN to kiwifruit dormant buds led to production of high levels of proline amino acid. They stated that elevated levels of proline in HCN-treated plants could be associated with a greater stimulation of the pentose-phosphate pathway, resulting in a greater percentage of budbreak and increased bud fruitfulness.

In conclusion, it can be stated that HCN is an effective compound for pistachio seed germination. Moreover, the role of ordinary chemical seed pre-treatments such as GA_3 and KNO_3 to improve pistachio seed germination was reconfirmed. Application of HCN also leads to precocious germination and early seedling development. This parameter may be useful for nurseries or fruit breeders as a useful measure to develop hybrid seedlings in a shorter time. Overall, although more experiments with other species should be carried out to confirm the results of the present research work, it can be said that utilization of HCN in seed testing experiments should be encouraged as an effective pre-treatment and dormancy breaking agent.

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