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Sarcotesta removal methods and GA_3 treatment on germination of *Punica granatum* L. seeds

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Resumo

Métodos de remoção da sarcotesta e aplicação de GA_3 na germinação de sementes de *Punica granatum* L. A propagação em *Punica granatum* é realizada, predominantemente, através da semente, ocorrendo baixa taxa de germinação, devido à dormência e à rápida perda do seu poder germinativo. Investigações relacionadas à remoção da sarcotesta em sementes dessa espécie são escassas, como também a utilização de tratamentos pré-germinativos de ácido giberélico (GA_3). Com isso, objetivou-se avaliar o efeito de métodos de remoção do arilo e o tratamento com GA_3, na germinação de sementes de *P. granatum*. As sementes foram extraídas de frutos maduros e submetidas a três métodos para extração da sarcotesta: 1) lavagem manual em peneira e água corrente; 2) repouso em solução de cal virgem, durante 24 h; e 3) fermentação em sacarose, por 72 h. As sementes foram divididas em duas porções iguais, uma imersa em solução de 200 mg L^{-1} de GA_3 e outra sem imersão em GA_3. A degomagem com cal virgem foi eficiente, removendo de modo fácil e rápido toda sarcotesta das sementes, proporcionando maior velocidade (4,26) e percentual (60%) de germinação às mesmas. O tratamento com cal virgem associado à aplicação de GA_3 promoveu um incremento no percentual de germinação (70%), contribuindo para melhor superação da dormência.

Palavras-chave: Ácido giberélico; Dormência; Sementes de romã

Abstract

Propagating *Punica granatum* is predominantly conducted using seeds, however, the germination rate is low due to seed dormancy associated with rapid loss in germination potential. Investigations related to sarcotesta extraction of pomegranate seeds are scarce, as well as pre-germination treatments with gibberellic acid (GA_3). Thus, the aim of this study was to evaluate the effect of three sarcotesta extraction methods and a gibberelic

acid (GA_3) treatment on the germination of *P. granatum* seeds. The seeds were removed from ripe fruits and submitted to the following sarcotesta extraction methods: 1) manual friction on sieve under running water; 2) immersion in quicklime solution for 24 h; and 3) sucrose fermentation for 72 h. The seeds were divided into two equal parts; the first portion was immersed in 200 mg L^{-1} of GA_3 solution and the second portion was not immersed in GA_3. The quicklime method was efficient and easily and quickly removed the sarcotesta from all seeds. This method resulted in a greater germination speed index (4.26) and germination percentage (60%). The quicklime method associated to the GA_3 treatment promoted an increase in germination percentage (70%) and contributed to dormancy breaking.

Key words: Gibberellic acid; Dormancy; Pomegranate seed

Introduction

The pomegranate tree (*Punica granatum* L., family Lythraceae) is a shrubby fruit and ornamental plant (CORRÊA, 1978) that has therapeutic properties (WERKMAN et al., 2008). The fruit has a fleshy pericarp and contains numerous irregular seeds that have a translucent sarcotesta, sclerotic mesotesta and testa composed of fleshy cells (BARROSO et al., 1999). The sarcotesta is a good source of sugars, vitamin C, vitamin B, pantothenic acid, potassium, antioxidants and polyphenols, and is also a reasonable source of iron (DEEPIKA; KANWAR, 2010). In addition, different parts (flowers, fruits and bark) of the species are used phytotherapeutically to treat several ailments, predominantly gastrointestinal problems (LANGLEY, 2000).

Pomegranate propagation is performed at large scale using seeds (BATISTA et al., 2011). However, difficulties in obtaining seedlings from seeds have been reported due to unsatisfactory plant establishment (MATERECHERA; SEEISO, 2013) caused by seed dormancy (RAWAT et al., 2010) and rapid loss of germination potential (LOPES et al., 2001; KANWAR et al., 2010).

Several factors must be considered when regulating the germination process and overcoming dormancy, such as the sarcotesta extraction method, storage, temperature effect, and balance between promoters and inhibitors (WARREN; BENNET, 1997). The sarcotesta extraction method is directly related to obtaining high seed quality (SILVA, 2012), should be done carefully to preserve the physiological quality of the seeds and can even be beneficial to the seeds (CARDOSO, 2011). For the seeds of some fruit species, the method should be specific and can be laborious and sometimes problematic for seeds that have mucilaginous substances. Thus, each species must be considered individually, according to the physiological characteristics (CARVALHO; NAKAGAWA, 2012).

Investigations about pomegranate sarcotesta extraction are scarce. Lopes et al. (2001) and Sousa et al. (2012) reported that fermenting seeds for 72 h resulted in greater viability and vigor, and was effective at removing the sarcotesta. In other species, pre-treatments are commonly used, such as immersion in hot water, hot air, mechanical or chemical scarification for dormancy caused by the seed coat (physical dormancy), and warm and/or cold stratifications that are usually applied for seed dormancy caused by embryo restrictions (morphophysiological dormancy); the latter causes hormonal balance changes, especially the levels of endogenous gibberellins and abscisic acid (LANDIS et al., 1996; FINCH-SAVAGE; LEUBNER-METZGER, 2006; TAKATA et al., 2014).

Gibberellins are regarded as germination promoters because they are involved in activating embryo growth, weakening the seed coat and endosperm layers that surround the embryo and restrict its growth, as well as mobilizing energy reserves (KUCERA et al., 2005; TAIZ; ZEIGER, 2009). They are also involved with protein synthesis and specific RNA during germination, both in dormancy breaking and control of hydrolysis reserves; in this sense, they stimulate hydrolysis synthesis as α-amylase that degrades starch, releasing energy for embryo development (TAIZ; ZEIGER, 2009).

Lopes et al. (2001), in their control that did not apply any treatment to improve the germination process,

observed a low germination percentage for pomegranate seeds. On the other hand, Rawat et al. (2010) obtained a substantial response with higher, faster, and uniform germination, as well as significant growth at the early seedlings stage, when using 25 and 30 days of cold stratification (5ºC) on pomegranate seeds. Despite this, Takata et al. (2014) did not observe a positive germination response for a gibberelic acid (GA_3) treatment on pomegranate seeds from a commercial orchard.

In an extensive study on pomegranate cryopreservation, Silva (2013) reports the importance of conducting studies that investigate the reaction of seeds using different methods of sarcotesta extraction and emphasizes eliminating dormancy caused by the seed coat. Thus, the aim of this study was to evaluate the effect of three sarcotesta extraction methods and a GA_3 treatment on the physiological quality of *P. granatum* seeds.

Material and Methods

The experiment was performed at the Seed Analysis Laboratory of the Universidade Estadual do Norte do Paraná, Bandeirantes, Brazil. Ripe fruits from domestic orchards were used, which were harvested from different plants. The seeds were manually removed, divided into two equal parts and submitted to three methods of sarcotesta extraction.

The first method consisted of manually washing the seeds with a sieve under running water, causing friction between the pomegranate seeds and steel mesh, for 10 minutes. The second method involved adding a solution of quicklime (CaO) and distilled water to the seeds, which was placed inside a beaker, at volumetric proportions of 3:2:5, respectively. The seeds remained in the solution for 24 h. For the third method, the seeds were fermented in a distilled water and sugar solution (10:1) at room temperature for 72 h (Lopes et al., 2001).

After each treatment, the seeds were washed under running water on steel mesh for 2 minutes and placed on an absorbent sheet to dry out at room temperature for three days. Following this, the seeds were divided into two equal portions. One portion was immersed in a solution containing 200 mg L^{-1} of gibberellic acid (GA_3), for three minutes, and the other portion was not immersed in the solution.

The germination test was carried out on germination paper (Germitest®), in the dark, with an alternating temperature of 20–30ºC (8–16 h). There were four samples of 25 seeds per treatment, previously immersed for three minutes in a fungicide suspension of 0.2% metalaxyl-m + fluodioxonil (Maxim XL®). The evaluations were performed at 7, 14, 21, 28, and 35 days after sowing by counting the germinated seeds; those with a radicle length equal or greater than two millimeters were considered germinated. From these data, we evaluated seed germination percentages weekly and at 35 days after sowing, when germination stabilization occurred. The total germination percentage and germination speed index (GSI) were evaluated according to Maguire (1962), as well as the percentages of normal seedlings, abnormal seedlings (BRASIL, 2009) and ungerminated seeds, based on the germination percentage.

The experimental design used for the germination test was completely randomized in a factorial of 3x2 (three treatments for sarcotesta extraction with and without the gibberellic acid treatment), using four replicates of 25 seeds per treatment. The data were subjected to an analysis of variance using the program ASSISTAT and the averages were compared using Tukey's test at 5% probability.

Results

According to the results, the quicklime extraction method was the most efficient among the sarcotesta removal methods, requiring only two minutes under running water to completely remove the sarcotesta. On the other hand, the fermentation method did not completely remove the sarcotesta; fragments surrounding the seeds were observed after two minutes under running water. The friction on sieve method took ten minutes under running water to reach the same result of the previous method (fermentation).

For germination percentage, germination speed index, percentage of normal seedlings and ungerminated seeds, there was a significant interaction between the sarcotesta extraction methods and the GA3 treatment (Tables 1 and 2).

Germination stabilized 35 days after sowing. The seeds submitted to the quicklime extraction were the first to germinate (10%), which occurred before seven days; of these, those with the GA3 treatment had a better result (20%). The seeds from the other treatments began germinating seven and 14 days after sowing.

The initial result of a higher germination percentage for the quicklime method with GA3 remained until the end of the test (70%). The same occurred for the germination speed index (5.9) and normal seedlings (65%); there was an increase of 18.4% for normal seedlings when treated with GA3 (Table 2). The fermentation and friction on sieve methods resulted in later radicle emission. Both methods had similar averages for some characteristics, as well as low values compared to those for the quicklime extraction. These extraction methods did not promote breaking the morphological dormancy and an increase in germination, even for those associated with the GA3 treatment. Instead, there were higher percentages (92% for fermentation and 86% for friction on sieve) of ungerminated seeds (Table 2).

There was no noticeable interaction between the factors for abnormal seedlings (Table 3). Similar values for the extraction methods and GA3 treatment

TABLE 1: Germination percentage (%) of *Punica granatum* seeds, for the sarcotesta extraction methods and GA_3 treatment at 7, 14, 21, and 28 days after sowing.

*Treatments	7 days		14 days		21 days		28 days	
	GA_3 concentration (mg L^{-1})							
	0	200	0	200	0	200	0	200
Friction on Sieve	0 aB	0 aB	3 aB	3 aB	12 aB	8 aB	21 aB	11 bB
Quicklime (CaO)	10 bA	20 aA	36 bA	54 aA	48 bA	62 aA	55 bA	67 aA
Fermentation	1 aB	0 aB	4 aB	1 aB	6 aB	4 aB	9 aC	6 aB
$LSD_{extraction\ methods}$	4.7		9.8		8.6		9.4	
$LSD_{gibberellic\ acid}$	3.9		8.1		7.1		7.7	
CV (%)	35.7		32.9		20.8		14.7	

* Equal letters, lowercase in the row and uppercase in the column, do not differ by Tukey's test at 0.05 probability level.

TABLE 2: Germination percentage (GP), germination speed index (GSI), percentage of normal seedlings (NS) and ungerminated seeds (US) of *Punica granatum* seeds, for the sarcotesta extraction methods and GA_3 treatment, 35 days after sowing.

Treatments	GP (%)		GSI		NS (%)		US (%)	
	GA_3 concentration (mg L^{-1})							
	0	200	0	200	0	200	0	200
Friction on Sieve	26 aB	14 bB	1,1 aB	0,6 aB	13 aB	7 aB	74 bB	86 aA
Quicklime (CaO)	60 bA	70 aA	4,3 bA	5,9 aA	55 bA	65 aA	40 aC	30 bB
Fermentation	11 aC	8 aB	0,6 aB	0,3 aB	6 aB	2 aB	89 aA	92 aA
$LSD_{extraction\ methods}$	10.7		0.7		10.2		10.9	
$LSD_{gibberellic\ acid}$	8.8		0.6		8.4		8.9	
CV (%)	14.2		18.5		21.2		8.5	

*Equal letters, lowercase in the row and uppercase in the column, do not differ by Tukey's test at 0.05 probability level.

were observed, indicating that the abnormalities did not act on the normal seedling percentage, but on the ungerminated seeds.

TABLE 3: Abnormal seedlings percentage (AS) of *Punica granatum* seeds, for the sarcotesta extraction methods and GA_3 pre-germination treatment, 35 days after sowing.

Treatments	AS
Friction on Sieve	10.3 ^{ns}
Quicklime (CaO)	5.8
Fermentation	6.3
GA_3 0 mg L^{-1}	8.3
GA_3 200 mg L^{-1}	6.5
$LSD_{\text{extraction methods}}$	5.5
$LSD_{\text{gibberellic acid}}$	3.7
CV (%)	38.3

^{ns}not significant by Tukey's test at 0.05 probability level.

Discussion

The quicklime extraction method is used for some species that have mucilage surrounding the seed. This method can be efficient, as observed in this study for pomegranate seeds, and is effective and promotes significant results when it is adequately applied. As evidence, there have been studies conducted, mainly with *Passiflora* ssp., where this method is commonly used to treat seeds before storage or sowing (WAGNER JÚNIOR et al., 2011; DOS SANTOS et al., 2012; LOPES et al., 2013; MAROSTEGA et al., 2013; 2015).

For some species, there are studies of extraction methods with quicklime as a treatment, which mostly compare manual friction between seeds and quicklime using a sieve under running water. This has been conducted with *Passiflora edulis* (PEREIRA; DIAS, 2000; AGUIAR et al., 2014), *Jacaratia spinosa* (FREITAS et al., 2011), and *Hylocereus undatus* (ALVES et al., 2012). In other studies, seed immersion in quicklime solution for 10 minutes was evaluated for *Passiflora edulis* (MARTINS et al., 2006) and *Passiflora alata* (OSIPI et al., 2011). For *Eugenia jambolana*, the seeds were mixed with quicklime inside a container for 10 minutes, which produced friction, and then washed (ARAÚJO et al., 2015).

Despite the variation in how quicklime was used in these studies, the results always had similar averages and the highest germination or emergence percentage compared to other treatments, except for *Eugenia jambolana*, which was intermediate (ARAÚJO et al., 2015). Investigations of quicklime to remove the sarcotesta of *P. granatum* seeds do not exist in the literature. The results of the present study verified that letting seeds rest for 24 h in a water and quicklime solution worked as a chemical treatment and promoted seed coat scarification and an increase in water permeability that, consequently, stimulated the germination process.

Materechera and Seeiso (2013) investigated techniques to break the morphological dormancy and improve the germination of fresh and dried pomegranate seeds. They observed that dried seeds had significantly higher water imbibition (9.6 versus 17.8 $mm.h^{-1}$) and germination percentage (25.3 versus 67.8%) than fresh seeds, and the efficiency of the techniques was in the following order: sulfuric acid, hot water, gibberellic acid, sandpaper, scalpel and control.

Silva (2013) evaluated the effect of moisture content variation on the physical properties of pomegranate seeds and observed that the porosity of seed mass increased by increasing the moisture content. Takata et al. (2014) concluded that seed immersion in water (control) is an effective method to promote germination because they did not notice a response for different GA_3 doses on pomegranate seed germination. On the other hand, Materechera and Seeiso (2013) reported that immersion in sulfuric acid had statistically better results than other methods, including immersion in a gibberellic acid solution. However, the authors found a significant correlation ($r = 0.83$, $p < 0.01$) between water imbibition and germination, affirming that the seed treatments promoted water imbibition and the germination process.

The use of quicklime in the present study had results similar to those obtained by Materechera and Seeiso (2013) for sulfuric acid. It should be noted that besides increasing germination, quicklime has proved

to be an efficient and viable method to remove the sarcotesta, since it is easy to handle, cheap, and easy to obtain. In contrast, the lower seed quality values obtained for the fermentation extraction corroborate those verified by Lopes et al. (2001), where pomegranate seeds submitted to 72 h of fermentation and drying had a low emergence percentage and emergence speed index (20% and 0.2, respectively).

Among investigations looking for methods to overcome the physiological dormancy of pomegranate seeds, those that submitted the seeds to a stratification process stand out (CERVELLI, 1994; OLMEZ et al., 2007; RAWAT et al., 2010; GOKTURK et al., 2012; MATERECHERA; SEEISO, 2013), since there is a lack of information about gibberellin treatments for this species.

Due to the fact that no difference among GA_3 doses (0; 100; 200; 300; 400 mg L^{-1}) was observed for seed germination, Takata et al. (2014) suggested that the hormonal balance of pomegranate seeds influences germination (which may explain why the GA_3 did not influence the germination process) and observed a high percentage for all treatments. However, the authors emphasize that the seeds were obtained from a commercial orchard, suggesting the genetic characteristic must have influenced their results.

On the other hand, Rawat et al. (2010) tested seed stratification on moistened turf layers at 5 °C and observed an increase in seed quality and initial seedlings; after 30 days they obtained 92% germination, versus 40% for the control. Studies combining a pre-germination treatment of sulfuric acid and stratification have found that the interaction can result in higher, faster and more uniform seed germination (OLMEZ et al., 2007; GOKTURK et al., 2012).

For example, the immersion of pomegranate seeds in sulfuric acid for 30 minutes associated with stratification at 4°C for 45 days promoted 61% germination versus 7% for only sulfuric acid immersion for 10 minutes and 5% for the control, which were both statistically different from the interaction (GOKTURK et al., 2012). These observations indicate that pomegranate seeds probably exhibit morphophysiological dormancy because of the positive germination response when the seeds are immersed in a substance that promotes external scarification.

In addition, treatments that cause changes in hormonal balance can further increase the germination percentage, as observed in the present study where there was a significant interaction between the factors that resulted in high seed quality values for the quicklime method associated with the GA_3 treatment. Now that the potential of these treatments has been verified, studies should be conducted to evaluate different quicklime dosages and immersion periods, as well as different treatments of gibberellic acid.

In conclusion, the quicklime method is efficient and easily and quickly removes all of the sarcotesta from *P. granatum* seeds, which results in a substantial germination speed index and germination percentage. Also, this method combined with the GA_3 treatment increases the germination percentage and contributes to dormancy breaking.

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