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Effect of Different Pre-Sowing Treatments on Germination of Persian Walnut (*Juglans Regia* L.) in Rukum (East) District, Nepal

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ABSTRACT

A field-based experiment was carried out in Rukum (East), Nepal, from February to June 2020, to assess the effect of different pre-sowing treatments on germination of Persian walnut. The experiment was carried out in a randomized complete block design with six treatments and four replications. The treatments included hot water treatment, chilling stratification only, cracking + Gibberellic acid (500ppm) followed by chilling stratification, cracking + Gibberellic acid (750ppm) followed by chilling stratification, Gibberellic acid (500ppm) + chilling stratification and Gibberellic acid (750ppm) + chilling stratification. The minimum days for germination (15.75 days) and highest germination (53.25%) were obtained when the combination of cracking with GA3 @ 750 ppm along with chilling stratification was done. The maximum shoot length (34.83 cm) was observed in the combination of cracking with GA3 @ 500 ppm followed by stratification but statistically similar shoot length (34.63 cm) was observed when cracking, application of GA3 @ 750 ppm followed by stratification was done. Cracking, treatment with GA3 @ 500 ppm followed by chilling stratification resulted in the highest shoot fresh weight (11.93 gm) and root fresh weight (10.77 gm) compared to the other treatments used. Thus, cracking along with treatment by GA3 @ 750 ppm followed by chilling stratification could be suggested to the walnut growers for better germination and a better morphological and physiological status of the rootstocks/seedlings.

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Introduction

Walnut (*Juglans regia* L.) is the most widespread nut tree in the world and it is commonly called the Persian walnut, English walnut or common walnut. It belongs to the family Juglandaceae. The area under walnut cultivation in Nepal is 2167 ha while the national average production and yield of walnut are 8934 mt and 4.12 mt/ha respectively (MoALD, 2019). Rukum district, on the other hand, has a production of 742 mt (MoALD, 2019).

The methods of propagation in walnut can be classified as sexual (seed) and asexual/vegetative methods (Thapa et al., 2021). Propagation by seeds is a conventional method to propagate walnuts. It is one of the most recognized and efficient methods which is widely applied for different fruit species. Although sexual propagation do not result in true to type plants, in breeding programs, it is inevitable for growing the hybrid seedlings (Gandev, 2009). The sexually raised plants are long lived, with extensive and powerful root system having wide and deep distribution in the soil. This accounts for persistent and adequate annual growth of absorbing roots thus helping them acclimatize easily in the environment. Also, rootstocks exhibit a great effect on the production efficiency, yield quality, adaptability, tree vigor, resistance to biotic (diseases and insects/pests) and abiotic stresses (salt tolerance and water logging) of scion cultivar. Besides, the main function of seedling rootstocks is to provide anchorage by growing deep into the soil and regulating the uptake of moisture and nutrients (Thapa, 2021; Farsi et al., 2018).

Vegetative propagation in walnut is more difficult, compared to most of the fruit species and this results in a rather low success rate of grafting (Thapa et al., 2021; Gandev, 2007). However, there are also some difficulties in using seeds to propagate walnut species. The seed dormancy and inconsistent seed germination create problems in the improvement programs and sometimes result in loss of hybrid vigor (Raufi et al., 2017). Poor seed

germination is the major limiting factor of walnut for large scale production and cultivation under cold arid conditions. In walnuts, the poor seed germination has been correlated with physiological dormancy, that is controlled by seed coat and embryo dormancy (Matilla and Matilla, 2008). Various methods are used to overcome seed dormancy depending on the walnut species and dormancy type. In Nepal, generally walnut cracking and chilling of walnut is practiced for ensuring a better germination. Therefore, analyzing the causes of dormancy and evaluating the methods of breaking dormancy to increase seed germination percentage and rate is necessary (Rajabiyan et al., 2007).

If proper germination percentage is ensured, we can grow a greater number of rootstocks as well as we can obtain more number of seedlings of high yielding variety seeds. Promoting the germination of walnut seeds is equally important in breeding programs and the use of germplasms (Hartman et al., 2001). With the introduction of new varieties, there is a need to ensure the most appropriate method of pre-sowing treatment of walnut seeds so that a better germination percentage can be achieved (Thapa et al., 2021). Therefore, analyzing the causes of dormancy and evaluating methods of breaking dormancy is necessary, in order to increase seed germination percentage (Rajabiyan et al., 2007). In this light, the current study was carried out to determine the most suitable pre-sowing treatment in walnut for ensuring a good germination percentage and better physiological and morphological status of walnut rootstocks and seedlings.

Materials and Methods

Experimental Site

The experiment was carried out in a private nursery, located in Syalapakha, Sisne Rural Municipality-08 under the command area of PM-AMP (Walnut-zone), Rukum (East) district, Nepal (Figure 1). The research site is located at a latitude of 28.66º North and a longitude of 82.49º East. The altitude of the place is 1200 meters above the mean sea level. The climate of Syalapakha is characterized by three distinct seasons; rainy monsoon (June-October), cool winter (November-February), and mild spring (March-May) (Ahamad, 2020). The average precipitation was 1.042 mm per day, the total incident solar radiation during the study period was 640.93 kW-hr/ m^2/ day and the average relative humidity was 40.27% (NASA-Power, 2020).

Treatment Details

There were six different pre-sowing treatments in the experiment. The general description about the treatments used is shown in Table 1.

Table 1. Treatments used in the experiment

S. No	Treatments Used
1.	Hot water (24 hours)
2.	Chilling stratification
3.	Cracking+ GA3(500ppm) followed by the chilling stratification
4.	Cracking+ GA3(750ppm) followed by the chilling stratification
5.	GA3(500ppm) + chilling Stratification
6.	GA3 (750ppm) + chilling stratification

Experimental Design

The experiment was laid out in a Randomized Complete Block Design (RCBD). The experiment comprised six treatments and each of the treatment was replicated four times. The treatment was randomized between the blocks. There were five rows per plot and in each row, five seeds were sown. The row-to-row distance was 30cm and seed to seed distance was 10cm.

Figure 1. Research site of pre-sowing study located at Syalapakha (Sisne Rural Municipality-08), Rukum (East), Nepal
Source: Thapa et al. (2021)

Pre-sowing Treatments

Fully mature seeds of Persian walnut (*Juglans regia* L.), hard-shelled local variety were collected from the walnut farm of Thapa Nursery, Syalapakha, Rukum (East). Seeds were selected, washed, float-checked, and air dried. Selected seeds were subjected to different pre-sowing treatment/s. The different pre-sowing treatments were used either alone or in combination as mentioned in the Table 1 above.

Hot water treatment

The seeds were dipped in the container containing hot water (boiled at 50-60°C). The container was kept safely inside the farm without any disturbances. Nearly about 25-30 seeds were taken at a time and dipped 3-4 times to complete the treatment.

Chilling stratification

The seeds were desiccated to about 12% moisture on a dry weight basis and then surface sterilized by soaking in 5% sodium hypochlorite solution for 10 minutes. Then they were subsequently rinsed thoroughly with sterilized water prior to germination or chilling. The chilling temperature was set to 4±1°C for 700-1500 hours. The seeds were arranged alternatively with sand at different multilayer (3-4 layers) and finally covered with moist bags moss and kept in the wooden box for up to 8 weeks. After arranging them properly, the irrigation was done by water can for 1 week. Then, it was irrigated as per the moisture conditions. At the bottom of the wooden box, tiny holes were kept for the drainage purpose. Once radical emergence was observed in 50% of the walnut seeds, it was considered the completion of the chilling stratification.

Cracking

After carefully selecting the seeds, they were partially cracked using walnut cracking machine. Care was taken that the seeds did not crack completely. After careful cracking, the seeds were ready to be subjected for different treatments to initiate the germination.

Treatment of seeds with GA_3 solution

The seeds were soaked in the GA3 solution for 24 hours. The two different solutions of GA3 were prepared (500ppm and 750ppm). We dissolved 0.5gm of the GA3 in 1L of water, to make a 500ppm GA3 solution and similarly 0.75gm of GA3 was dissolved in 1L of water to make a 750ppm GA3 solutions. For uniform solubility, ethanol (90% pure) was used along with distilled water.

Field Operations

Tillage and Land Preparation

The land was prepared by single primary tillage and 2-3 cross secondary tillage followed by leveling of land. All the weed and plant residues were removed to make the field clean. FYM was well mixed into the soil during tillage operations. The land was prepared 15 days before sowing the walnut seeds.

Planting

The sowing was done on 17th of February, 2020. Single seed was sown in each hill. The distance from row to row was kept 30 cm and the distance from seed to seed was 10 cm. There were five rows per plot and in each row five seeds of walnut were sown.

Fertilizers and irrigation

Fertilizers were applied as per the recommendations given by AITC (2019). 50 kg of FYM was applied during the seedbed preparation while 434.78 grams of DAP, 699.43 grams of Urea and 333.33 grams of MOP were applied in the field, at the time of sowing. Light irrigation was done, generally 2-3 times a week.

Weeding

Regular hoeing of field was done up to 30-35 days of sowing with the help of a spade. Manual hand weeding was done after that. While hoeing and weeding, care was taken to not injure the seeds.

Data Collection

The biometrical observations were recorded from inner five randomly selected plants of each plot. The morphological and physiological traits were assessed from the sample plants. The following parameters were assessed through the data collection.

The germination percentage was calculated taking all the seeds sown in the plot as total.

Seeds were considered to be germinated when the radicle reached half the length of the seed. At the end of germination period (Eight week), the germination percentage (GP) was calculated using the following formula as given by Copeland and McDonald (2001):

$$GP = \sum G/N \times 100$$

Where GP is the germination percentage, G is the numbers of germinated seeds and N is the total number of seeds.

The days taken for germination in each of the pre-sowing treatment were observed and recorded.

At the end of experiment (3 months of sowing), seedlings were cut at soil surface and the roots were washed to make them free of soil.

Shoot length was taken with the help of a scale. It was measured in (cm).

Root length was taken with the help of a scale, and measured in (cm).

Fresh weight of the shoot was taken with help of a weighing balance.

Fresh weight of the root was taken with help of a weighing balance.

Data Analysis

The data collected were refined and entered in MS-Excel sheet. The data were analyzed to draw meaningful inferences by using the 'agricolae' package of statistical software R Studio Version 4.0. The mean comparison of the different parameters like germination percentage, days to germination, shoot length, root length, shoot and root fresh weight were done using DMRT (Duncan's Multiple Range Test).

Results

Days to Germination

From the table below, it can be observed that the seeds treated with the hot water required significantly higher number of days to germinate (55 days), followed by the seeds that were kept in chilling stratification (44.25 days). Furthermore, the cracked seeds treated with GA3 @750 ppm solutions, that were kept in chilling stratification, required significantly lowest number of days to germinate

(15.75 days). Seeds treated with GA3 @500 ppm and @750 ppm solution followed by chilling stratification showed statistically similar results with each other for the days to germinate.

Germination Percentage

Significant results were observed under different pre-sowing treatments for the germination percentage of walnut. The results have been shown in the Figure 2. From the graph, it can be devised that chilling stratification of the cracked walnut seeds along with dipping in the 750ppm of GA3 solution showed the highest germination percentage (53.25%) followed chilling stratification of the cracked walnut seeds dipped in the 500ppm GA3 solutions (51.75%). Both the treatments were statistically similar with each other for germination percentage. The results were then followed by chilling stratification of the walnut seeds that were dipped in the 750ppm GA3 solutions and seeds dipped in the 500ppm GA3 solutions followed by chilling stratification only. Lowest germination percentage (44%) was observed in the seeds where hot water treatment was done.

Table 2. Effect of different treatment on days to germination in Syalapakha, Rukum (East) during 2020

Treatments	Days to germination
Hot water	55.25[a]
Chilling stratification	44.25[b]
Cracking + GA3 @ 500 ppm + chilling stratification	20.50[d]
Cracking + GA3 @ 750 ppm + chilling stratification	15.75[e]
GA3 @ 500 ppm + chilling stratification	27.75[c]
GA3 @ 750 ppm + chilling stratification	26.75[c]
SEm (±)	15.046
$LSD_{0.05}$	3.062
F-$test_{0.05}$	***
CV (%)	6.409

*, **, and *** represent significance at 5%, 1% and 0.1% level, respectively. NS=non-significant. Means followed by common letter(s) within column are not significantly different with each other based on DMRT test.

Shoot and Root Length

The highest shoot length or shoot growth (34.825 cm) was noticed under combination of the cracked seeds with GA3 @500 ppm under chilling stratification followed by the combination of the cracked seeds with GA3 @750 ppm under chilling stratification i.e., 34.625 cm. Both of the treatments showed statistically similar results with each other. GA3 @ 500 ppm under chilling stratification resulted in a shoot length of 24.325 cm, followed by the other treatments, which were statistically similar with each other.

The highest root length (25.35 cm) was observed on application of GA3 @500 ppm in the cracked seeds followed by chilling stratification followed by cracked seeds+ GA3 @ 500 ppm+ chilling stratification which were statistically similar with each other. The combination of cracking with GA3 @ 750 ppm under chilling stratification and GA3 @ 500 ppm under chilling stratification were statistically similar with each other, followed by the seeds kept in the chilling stratification. The lowest root length (13.35 cm) was observed in case of seeds treated with hot water.

Shoot and Root Fresh Weight

The maximum shoot fresh weight (11.930 gm) was recorded in the combined application of the cracked seeds, gibberellic acid @ 500 ppm and chilling stratification. The maximum shoot fresh weight was followed by treating the cracked seeds with GA3 @ 750 ppm and chilling stratification. It was followed by the seeds treated with GA3 @ 500 ppm kept under stratification, treatment with GA3 @ 750 ppm and hot water treatment. The lowest shoot fresh weight was recorded in case of seeds kept in chilling stratification only.

The maximum root fresh weight (10.77 gm) was observed in the combined application of the cracked seeds, gibberellic acid @ 500 ppm and chilling stratification followed by the seeds treated with GA3 @ 500 ppm and chilling stratification. Root fresh weight of the seeds kept in chilling stratification was statistically similar with cracked seeds treated with GA3 @ 750 ppm under stratification. The lowest root fresh weight was recorded in the seeds treated with hot water.

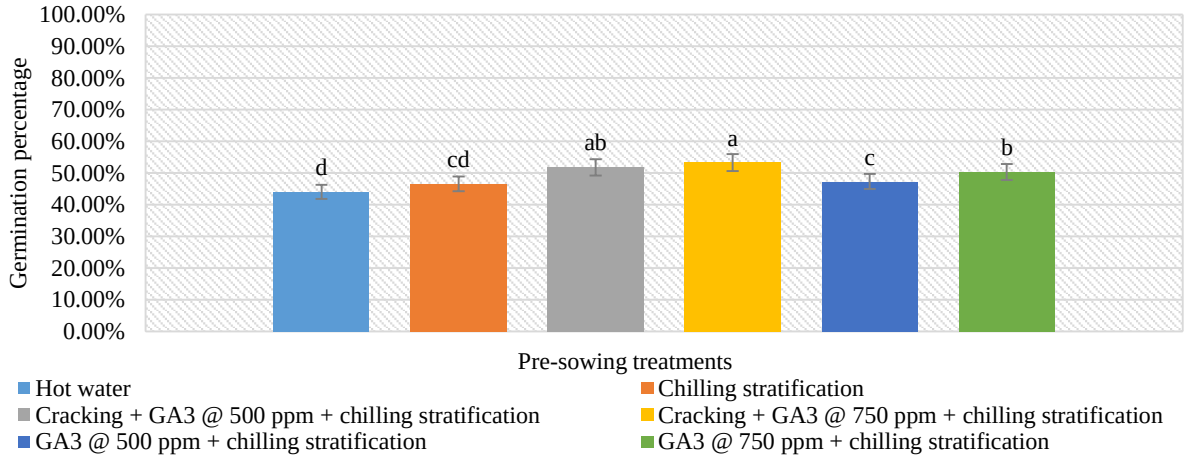

Figure 2. Effect of different pre-sowing treatments on germination percentage in Syalapakha, Rukum (East) during 2020

*Note: Means followed by common letter(s) along the graph are not significantly different with each other based on DMRT test.

Table 3. Effect of different treatment on shoot length in Syalapakha, Rukum (East) during 2020

Treatments	Shoot length (cm)	Root length (cm)
Hot water	21.550^{c}	13.350^{e}
Chilling stratification	20.375^{c}	19.775^{d}
Cracking + GA3 @ 500 ppm + chilling stratification	34.825^{a}	25.350^{a}
Cracking + GA3 @ 750 ppm + chilling stratification	34.625^{a}	24.575^{ab}
GA3 @ 500 ppm + chilling stratification	24.325^{b}	24.100^{bc}
GA3 @ 750 ppm + chilling stratification	21.475^{c}	23.250^{c}
SEm (±)	6.734	4.542
$LSD_{0.05}$	1.308	0.947
F-test$_{0.05}$	***	***
CV (%)	3.314	2.891

*, **, and *** represent significance at 5%, 1% and 0.1% level, respectively. NS=non-significant. Means followed by common letter(s) within column are not significantly different with each other based on DMRT test.

Table 4. Effect of different treatment on shoot and root fresh weight in Syalapakha, Rukum (East) during 2020

Treatments	Shoot fresh weight (gm)	Root fresh weight (gm)
Hot water	5.385^{d}	4.140^{e}
Chilling stratification	4.845^{e}	6.932^{d}
Cracking + GA3 @ 500 ppm + chilling stratification	11.930^{a}	10.770^{a}
Cracking + GA3 @ 750 ppm + chilling stratification	10.485^{b}	7.156^{d}
GA3 @ 500 ppm + chilling stratification	6.237^{c}	8.457^{b}
GA3 @ 750 ppm + chilling stratification	5.040^{de}	7.627^{c}
SEm (±)	3.082	2.161
$LSD_{0.05}$	0.387	0.323
F-test$_{0.05}$	***	***
CV (%)	3.509	2.857

*, **, and *** represent significance at 5%, 1% and 0.1% level, respectively. NS=non-significant. Means followed by common letter(s) within column are not significantly different with each other based on DMRT test.

Discussion

Early germination (15.75 days) and best germination (53.25%) was observed when combination of cracking with GA3 @ 750 ppm chilling stratification was used. The maximum germination might be due to the fact that GA3 involved in the activation of cytological enzymes which stimulated α - amylase enzyme that converted insoluble starch into soluble sugars (Babu et al., 2010) and early germination might be due to the fact that, GA3 played important role in two stages of germination one at initial enzyme induction and other in activation of reserve food mobilizing system which helped in enhancement of germination (Jha et al., 1997). In the present studies, it had been observed that gibberellic acid was required in relatively lower concentration with stratification and scarification for the maximum germination. The inability of walnut seeds to germinate might be due to the hard seed coat. As the scarification treatment given to the seed helped in uptake of water, growth hormones and air which was required for seed germination (Finch-Savage and Leubner-Metzger, 2006; Pallavi et al., 2014). Prechilling stratification might have a significant effect on seed dormancy because, at low temperature, more oxygen is dissolved in water making more oxygen available for the embryo (Young & Young, 1992).

According to Hassan and Fetouh (2014), stratification affected metabolic processes including changes in hormones, disappearance of ABA, activation of GA3 and initiation of germination. Without seed stratification, pretreatment with GA3 had no effect on the germination percentage of seeds, since few seeds germinated with GA3 application alone, indicating that GA3 was unsuccessfully substituted for cold stratification. On the other hand, the apparent response to GA3 pretreatment when stratification was combined with it, suggested a synergistic effect for both.

From our study, the least germination percentage and maximum days taken for germination was observed in hot water treated seeds. This might be due to the fact that, the ability of hot water to separate the columnar macro-sclereid cells through thermal expansion (Teketay, 1998) was ineffective in permitting water to penetrate the seeds. Our experiment was however in contrast with Negi et al. (2017) where they reported early germination at 12.67 days and best germination of 75.88% when GA3 was used as pre-sowing treatment. This might be due to the fact that seedlings varieties used in their research trial were of better quality and due to the availability of optimum temperature and humidity.

The maximum shoot length (34.825 cm) was noticed under combination of cracking with GA3 @ 500 ppm followed by stratification and similarly, shoot length (34.625cm) was also observed highest with the combination of cracking with GA3 @ 750 ppm followed by stratification. It might be due to the effect of GA3 and stratification on enhancing growth due to the solubility of fats and sugars caused by stratification plus the increased in gibberellins synthesis. In addition, the improving effect of GA3 and stratification on seed germination might had reflected on enhancing the shoot parameters. These results are in agreement with Dahkaei (2009) on *Danae racemosa*, Gokturk et al. (2010) on *Punica granatum* and Hassan and

Fetouh (2014) on seeds of *Magnolia grandiflora*. Similar observations were recorded by Mathur et al. (1972) in peach and apricot seedlings. The GA3 hormone increased cell size by stimulating the cell wall to release and transmit its calcium into the cytoplasm that provided a condition for absorption of water and cell growth and in stratification, endosperm was disrupted permitting embryo growth. On the other hand, low temperatures stimulated the breakdown of proteins into soluble nitrogenous compounds and formation of the amino acids' glycine and arginine, which were beneficial for embryo growth (Baskin and Baskin, 2001; Razavi and Hajiboland, 2009).

The highest root length (25.350 cm) was recorded with the application of cracked seeds + Gibberellic acid @ 500 ppm + chilling stratification. It might be due to the reason that the shoot growth resulted in production of photosynthates which were translocated through phloem to the root zone and was responsible for increase in root length. Parvin et al. (2015) who reported maximum root length and root area of walnut with combination of gibberellic acid and stratification, the effect of GA3 and stratification on root parameters followed the same trend as on the shoots. The positive effect of GA3 and stratification on root parameters might be explained through the role of GA3 and stratification in enhancing gibberellins synthesis which also leads in increased growth of root, root branching and overall increase in root fresh weight (Penfield et al., 2005).

The maximum shoot fresh weight (11.930 gram) and root fresh weight (10.770 gram) was found in the combined application of cracking with GA3 @ 500 ppm under stratification. This promotion could be explained through the role of stratification in enhancing gibberellins synthesis which also leaded to increase in the growth and root branching and overall increased roots fresh weight. Similar observations were recorded and confirmed by Panwar,(2010) in Jatropa, Fariman et al. (2015) in *Echinacea purpurea* and Parvin et al. (2015) in black walnut.

Conclusion

The study showed cracked seeds dipped in GA3 @750 ppm solution followed by chilling stratification led to the early germination, better germination percentage and a better morphological and physiological status. It could be suggested to the walnut growers of Rukum (East) district and to the growers of similar agroclimatic conditions to use the pre-sowing treatment i.e., cracking followed by GA3@750 ppm and chilling stratification. The results could be useful for nurserymen for producing a better number of rootstocks with better morphological and physiological status.

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Conflict of Interest

The authors indicate no conflict of interest for this work.

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