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Imbibition and germination of fresh chincuya (*Annona purpurea* Moc & Sessé Dunal) seeds, by effect of gibberelic acid and pH of the soaking water.

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Abstract: Germination is influenced by several factors, among them pH. Two experiments were conducted to determine the effect of imbibition-water pH on the water absorption curve of chincuya seeds and germination. The results were contrasted to the known effect of gibberellic acid imbibition. In the first year of fruit collection and evaluation, imbibition curves were constructed per treatment. The seeds of this evaluation were divided into two lots: the first at the initial moisture content (39.9 %) and the second at the moisture content of 34.1 % after three days of drying in the laboratory. The seeds were imbibed for 84 h in distilled water at three pH levels (5, 7, and 9) and kept in a water bath at 30 °C. In the second year of collection and evaluation, the pH treatments were repeated, and an imbibition treatment with gibberellic acid at 350 mg L^{-1} (pH 3.9) was added. A control treatment contained distilled water at pH 6.1. For both years, treated seeds were incubated on paper towels and placed under controlled conditions at a 12-hour photoperiod and alternating day and night temperatures (30 °C:25 °C). The three imbibition curves showed similar behavior and no statistical significance. Seeds with a higher moisture content did not gain weight after imbibition. The imbibition curves increased constantly in the seeds at 34.1 % moisture, and weight gain reached almost 46 % with the pH 9 treatment. However, there were no statistical differences among treatments. The highest rate of water absorption occurred in the first six hours in the open-air, dehydrated seeds, and after 72 hours, the weight of the seeds did not increase. Average daily germination kinetics indicated a slow and poor germination process. Gibberellic acid significantly promoted germination, causing 43 % germination. The absolute control reached 5.7 %, while the rest of the treatments did not promote germination.

Keywords: water absorption curve, Phase I, physiological dormancy, moisture content.

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Embebição e germinação de sementes de chincuya (*Annona purpurea* Moc & Sessé Dunal) sob efeito de ácido giberélico e pH da água em imersão

Resumo: A germinação é influenciada por diversos fatores, entre eles o pH. Para conhecer o efeito do pH da água de embebição na curva de aquisição de água das sementes de chincuya, assim como na germinação e compará-lo com o efeito conhecido da embebição em ácido giberélico, foram realizados dois experimentos. Em um primeiro ano de coleta de frutos, construíram-se as curvas de embebição com os tratamentos. As sementes desta avaliação foram divididas em dois lotes: o primeiro com o conteúdo de umidade inicial (39.9%), e o segundo com sementes secas por três dias em laboratório (34.1% de umidade). As sementes foram embebidas por 84 horas em água destilada com três diferentes níveis de pH (5, 7 e 9) e foram mantidas em banho-maria a 30°C. No segundo ano de coleta foram repetidos os tratamentos de pH e acrescentado o tratamento de embebição com ácido giberélico a 350mg L^{-1} (com pH 3.9) e a testemunha com água destilada (pH 6.1). Nos dois anos de avaliação, as sementes receberam os tratamentos, foram incubadas em toalhas de papel e mantidas em condições controladas de temperaturas alternadas de 30°C dia e 25°C noite, com 12h de fotoperíodo. Observou-se que as três curvas de aquisição de água mostraram comportamento semelhante e sem significância estatística; as sementes não aumentaram de peso depois da imersão, pois o conteúdo inicial de umidade era elevado. Nas sementes com 34.1% de umidade, as curvas de aquisição de água tiveram aumento constante e o peso das sementes aumentou até cerca de 46% com o pH 9. Não houve diferença estatística entre os tratamentos. A maior velocidade de aquisição de água ocorreu nas primeras seis horas nas sementes secas ao ambiente e depois de 72 horas o peso das sementes não aumentou. A cinética da germinação média diária apresentou um lento e escasso processo germinativo. O ácido giberélico aumentou significativamente a germinação, promovendo 43% de germinação, enquanto com a testemunha absoluta se alcançou 5.7%. Com os demais tratamentos não se observou germinação.

Termos de indexação: curva de aquisição de água; Fase I da germinação; dormência fisiológica; conteúdo de umidade.

Introduction

In germination studies, the first problem is accurately determining the time of seed imbibition as several factors condition it. It is common to use 24 hours of imbibition, disregarding that imbibition is affected by the reserve substances of the seed. For example, carbohydrates hydrate quickly, while fats require more time (MATILLA, 2000). The term germination includes several processes that lead to the initiation of embryo growth and radicle emergence. Many changes must occur, and imbibition is the first step. The processes necessary to hydrate and reactivate cellular enzymes require much more respiratory energy than is required to keep the seed dry (BEWLEY; BLACK, 1978). Since enzymes are biochemical catalysts, they play a critical role in cellular metabolism. The factors affecting enzyme activity are temperature, pH, and available reserve substances (AZCÓN-BIETO; TALÓN, 2000; TAIZ; ZEIGER, 2010). Most biological processes are susceptible to pH because changes in hydrogen ion content affect the ionic state of biological molecules (KARP, 2011). Enzymes function best within a specific pH range, and just as with temperature, extreme pH values may denature enzymes (TAIZ; ZEIGER, 2010).

Chincuya seeds are mainly lipidic (VIDAL-LEZAMA et al., 2019), and in this type of seeds, the degradation of oils starts with lipases that catalyze the hydrolysis of triacylglycerols and release fatty acids and glycerol (QUETTIER; EASTMOND, 2009). Nielsen and Liener (1984) demonstrated in *Phaseolus vulgaris* L. that the activity of two enzymes related to protein degradation increased as a function of pH. For α- N-benzoyl-DL-arginine-p-nitroanilide, pH 8.2 was better, whereas, for azoasein, it was pH 5.5.

Water adsorption in a dry seed occurs in three stages. The initial rapid Phase I activates respiratory metabolism and transcriptional activities. The duration depends on the hydratable compounds in the seed, testa permeability, size, weight, and anatomy (MATILLA, 2000; MANZ et al*.,* 2005). Protein-rich seeds absorb more water than fat-rich seeds. The rate of water uptake towards the end of this phase is temperature-dependent and is accompanied by increases in respiratory rate and light sensitivity in some species. These observations suggest that water uptake during imbibition is not passive but active (FINCH-SAVAGE, 2004). The second phase (Phase II), known as the plateau phase, slows down the rate of water absorption due to the change in seed water potential; hydration activates respiratory metabolism and releases energy. Metabolism resumes immediately (MATILLA, 2000). Newly synthesized, repaired, and existing mRNAs are activated, and protein synthesis begins, so hydrolytic enzymes initiate numerous reactions necessary to digest reserves (MANZ et al*.,* 2005). Seed hydration also causes the hydrolysis of some "conjugated" forms of phytohormones and their transformation into "free" ones, which, together with newly synthesized ones, initiate the hormonal action of the development program leading to seed germination (MATILLA, 2000). Phase III occurs after germination and water absorption increases; the embryo axes elongate, and root protrusion is displayed (MANZ et al., 2005).

On the other hand, gibberellins play a vital role in germination. Bewley and Black (1994) point out that they are synthesized in imbibed seed embryos and induce hydrolytic enzyme synthesis to weaken the seed coat, mobilize seed nutrient storage reserves, stimulate embryo expansion and hypocotyl elongation, activate meristems, and produce new shoots and roots. It is known that dormancy release is characterized by the capacity for increased abscisic acid degradation and enhanced gibberellin biosynthesis that promotes germination (FINCH-SAVAGE; LEUBNER-METZGER, 2006). FERREIRA et al. (2011, 2014) and VIDAL-LEZAMA et al. (2015a,b) have described the erratic and poor germination of *A. purpurea* and have shown the positive effect of gibberellins. This research measured the impact of the pH in imbibition water on the water absorption curve of chincuya seeds and germination. Also, we contrasted these results against results from imbibition in gibberellic acid, under the hypothesis that imbibing seeds at different pH levels would help the germination process in the same way as gibberellic acid.

Materials and Methods

Plant material. Seeds were extracted from mature fruits collected in two consecutive years from Las Salinas, Municipio, Chicomuselo, Chiapas, Mexico. The initial seed moisture in the first year was 39.9 %, while in the second year, it was 34.1 %. In the first year of collection, two batches of seeds were handled. One had the initial moisture content (seeds without dehydration), and the other batch contained seeds kept in the laboratory environment for three days (ambient average temperature of 22 °C and relative humidity of 32 %) at an estimated moisture content of 31.8 %.

Evaluation of seed weight loss. In four sets of ten seeds from the first collection, the weight at 0, 24, 48, and 72 hours after extraction was measured, and the weight loss percentage in grams was calculated.

Treatments applied. Three pH and gibberellic acid levels were tested. The pH levels 5, 7, and 9 were prepared by adding 1 N hydrochloric acid (HCl) and 0.5 N sodium hydroxide (NaOH), as needed, to distilled water. The gibberellic acid (Merk®) solution had a concentration of 350 mg L^{-1} at pH 3.9. An absolute control was also included, i.e., imbibition of chincuya seeds in distilled water at pH 6.1.

Water absorption curves. These were constructed by imbibing the seeds from the first collection for 84 hours in the corresponding treatment. For this, they were introduced in gauze bags and glass jars in a water bath at 30 °C, in 4 replicates of 10 seeds each. In the first evaluation, the weight of the embedded seeds per replicate was recorded every 12 hours, and they were again immersed in the same imbibition water. The seeds were not aerated except when removed from the water to be weighed.

Germination curve. In the second evaluation, the germination curve was generated with the averages of 10 replicates of 20 seeds each. Every 6 hours, the weight of the imbibed seeds was obtained, the pH was recorded, and the treatments were renewed; with this procedure, the seeds were aerated for about 10 min. The imbibed seeds were placed on blotting paper towels, which were moistened to saturation with distilled water, rolled and placed in a semi-closed plastic bag, watered with a manual sprinkler and placed in a growth chamber (Shel Lab LI15 ®) with alternating temperatures of 30 °C in the day and 25 °C in the night, in 12 hours of photoperiod. During the 40 days of the experiment, paper towels were changed three times, and the seeds were imbibed in 10 % (v/v) sodium hypochlorite (NaClO) solution (Chloralex®) for 3 min and then rinsed once with distilled water. Germinated seeds showed visible radicles (approximately 1 mm). Dormant seeds maintained the same initial appearance until the end of the test, while dead seeds showed evident signs of deterioration, such as loss of firmness and foul odor. The percentages of germination, dormant, and dead seeds were calculated; the imbibition speed was also calculated, considering the weight gained (g) in 6 hours. To assess the effect of the treatments on the response variables, the analysis of variance of a completely randomized design and Tukey's test were used, with a significance level of 5%. Additionally, data on seed numbers were examined by logistic regression to confirm the significance of the analysis of variance. The transformed data and those obtained via logistic regression were equally significant using the SAS statistical program (SAS Institute, 2000).

Results and Discussion

Evaluation of weight loss. It was observed that, at 24 hours of exposure to the ambient, the seeds had lost 24. 2 % of the initial weight; at 48 hours, 29.1 %, and at 72 hours, they lost 30 % of the initial weight. The testa is known to be permeable (VIDAL-LEZAMA et al., 2008; 2015a and FERREIRA et al., 2014), but the water loss rate is still unknown. After 72 h of being kept in laboratory conditions (22 °C and 32 % relative humidity), the seed moisture matches the environmental moisture. This behavior shows the water regulation by the seed in response to its environment. There is no information regarding the anatomical structure or composition of the testa of chincuya, but the data suggest that it is a very porous testa. The protective wax of the seeds is present in other species of anonas but not in *A. purpurea*. In *A. purpurea*, the testa is fibrous, striated, lignified and rough to the touch (VIDAL-LEZAMA et al., 2023); in the seeds of *A. macroprophyllata, A. muricata, A. reticulata, A. cherimola,* and *A. squamosa* (VIDAL-LEZAMA et al., 2015b) the protective wax on testa shows by the smooth texture and gloss. In *A. diversifolia*, Ferreira et al. (2014) demonstrated, by sealing seeds with kerosene in the hilum and ventral suture, that water entry into the seed is not hindered as it enters mainly through the testa, the raphe-anti raphe suture and the micropylar zone.

Effect on water imbibition curves.

Imbibition curve of chincuya seeds without dehydration (wet). The absence of significant differences between treatments is shown in Table 1, while Figure 1 shows the water absorption curves obtained with freshly collected and undehydrated seeds with similar behavior. It was observed that seeds imbibed in water at pH 5 and 7 at 12 h and those at pH 9 at 24 h decreased their weight and then recovered it (Figure 1).

Table 1. Effect of imbibition for 84 hours of freshly collected, undehydrated chincuya seeds in distilled water at different pH levels.

pH Treatment	Initial Weight (g)	Final Weight (g)	Percentage Gain
5	13.111 NS [z]	13.197 NS	0.65 NS
7	13.157	13.275	0.88
9	13.894	13.970	0.54
CV (%)	0.055	0.546	0.350

[z] NS. Means without statistical difference according to Tukey's test ($p \leq 0.05$).

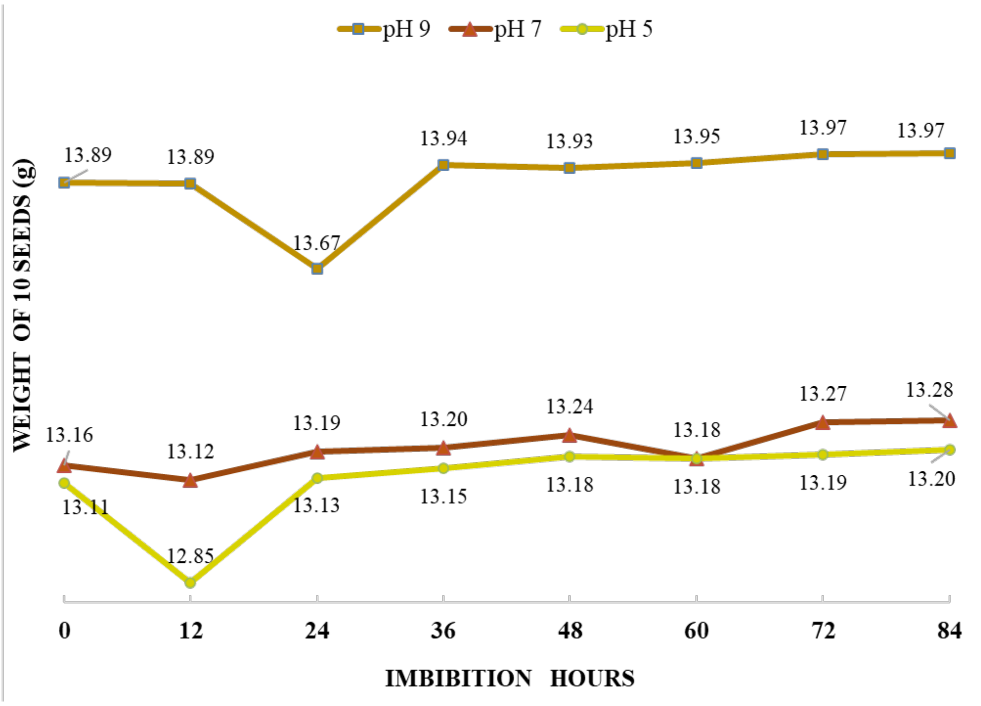

Figure 1. Imbibition curves of freshly collected wet chincuya seeds immersed in distilled water at different pH levels.

The loss of solutes explains this decrease in initial water uptake. Villa-Hernández et al. (2013) point out that the most important event during imbibition is probably the reorganization of cell membranes since it occurs before other events and is a precondition for most cellular processes. Adopting the hexagonal Phase II of the membrane contributes to the leakage of solutes from the substantial cytoplasm after hydration. Membranes must rapidly reorganize from a hexagonal Phase II to a lamellar phase to restore normal function and terminate leakage of cellular components. DNA damage repair begins early in Phase I and ends in Phase II. Initiation of nutrient reserve mobilization occurs early in Phase II, and solute leakage must stop by the end of Phase I; otherwise, germination may not happen (BEWLEY, 1997). Seeds gained virtually no weight after 84 hours of imbibition, and the calculation of weight gain shows so (Table 1). Neither treatment resulted in a significant increase in final weight, which implies that the seeds were completely saturated with water (37 %, two-year average) at the beginning of the treatment.

Imbibition curve of seeds kept in the open air for 72 hours. After 84 hours of imbibition (Table 2, Figure 2), the seeds gained weight by almost 46 % (pH 9). Azcón-Bieto and Talón (2000) point out that the hydration of dry and orthodox seeds depends on the water potential of their cells. An aqueous solution at atmospheric pressure has a negative water potential. Water molecules diffuse in response to water potential gradients from higher to lower potentials; thus, dry seeds imbibe.

Table 2. Effect of imbibition for 84 hours of freshly collected chincuya seeds exposed to open air for 72 hours in distilled water at different pH levels**.**

Treatment pH	Initial Weight (g)	Final Weight (g)	Percentage Gain (%)
5	8.739 NS[z]	12.495 NS	42.984 NS
7	8.933	12.513	40.074
9	8.554	12.458	45.642

[z] NS. Means without statistical difference according to Tukey's test ($p \leq 0.05$).

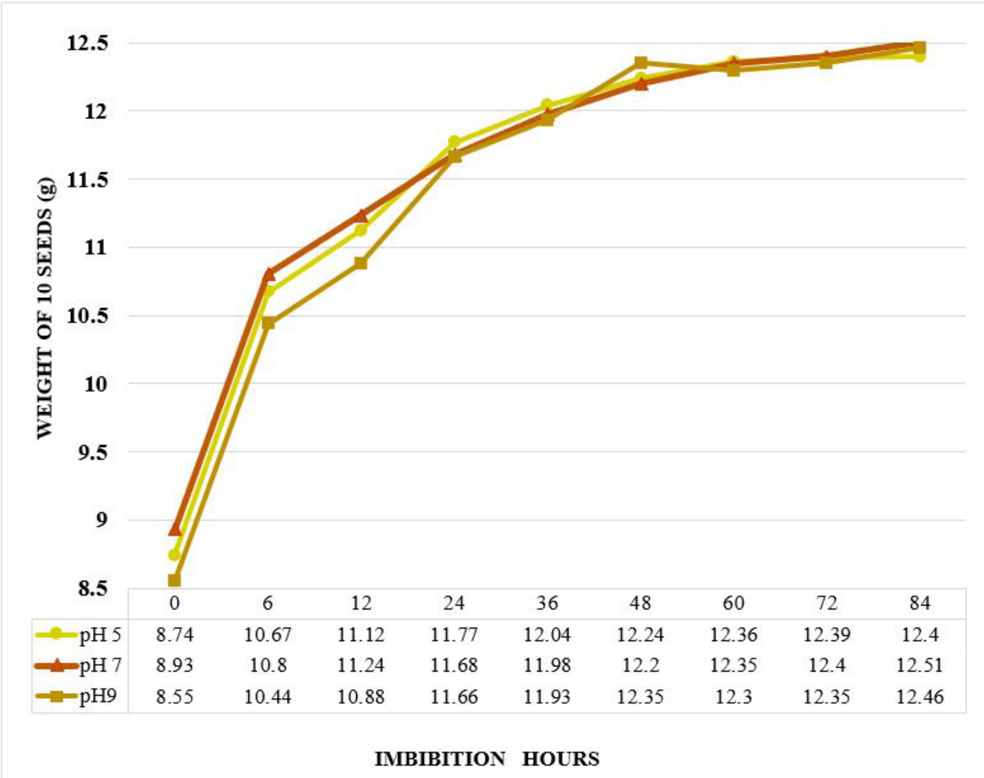

Figure 2. Imbibition curves of freshly collected chincuya seeds exposed to open air for 72 hours and imbibed in distilled water at different pH levels.

Ferreira et al. (2014) found that both *A. macroprophyllata* and *A. purpurea* had slow imbibition (Phase I) of 50 and 70 hours, respectively, while Costa et al. (2017) determined that the duration of imbibition in *A. emarginata* was 122 hours. VIDAL-LEZAMA et al. (2015a) determined the distilled water absorption curves of seeds of *A. macroprophyllata* and *A. purpurea* stored for five months and of *A. squamosa* at six months of storage; seeds of *A. purpurea* gained 35 % weight and stopped imbibing at 72 hours. It was observed (Table 2) that neither the initial nor final seed weights were significantly different between treatments.

The highest rate of water absorption occurs in the first six hours (Figure 3) since, as time progresses, the water potential of the cells is modified, generating less tension. Between 36 and 42 hours, there is a slight increase in the imbibition rate, probably indicating the repair of cell membranes. Different authors agree (MATILLA, 2000; FINCH-SAVAGE, 2004) that the rapid rate of initial imbibition may cause cell damage. The entry of water into the seed depends on the low water potential of the dry seed; however, it should be considered that storage components may hydrate at different rates. Lipids are hydrophobic; consequently, oilseeds have a slower imbibition curve.

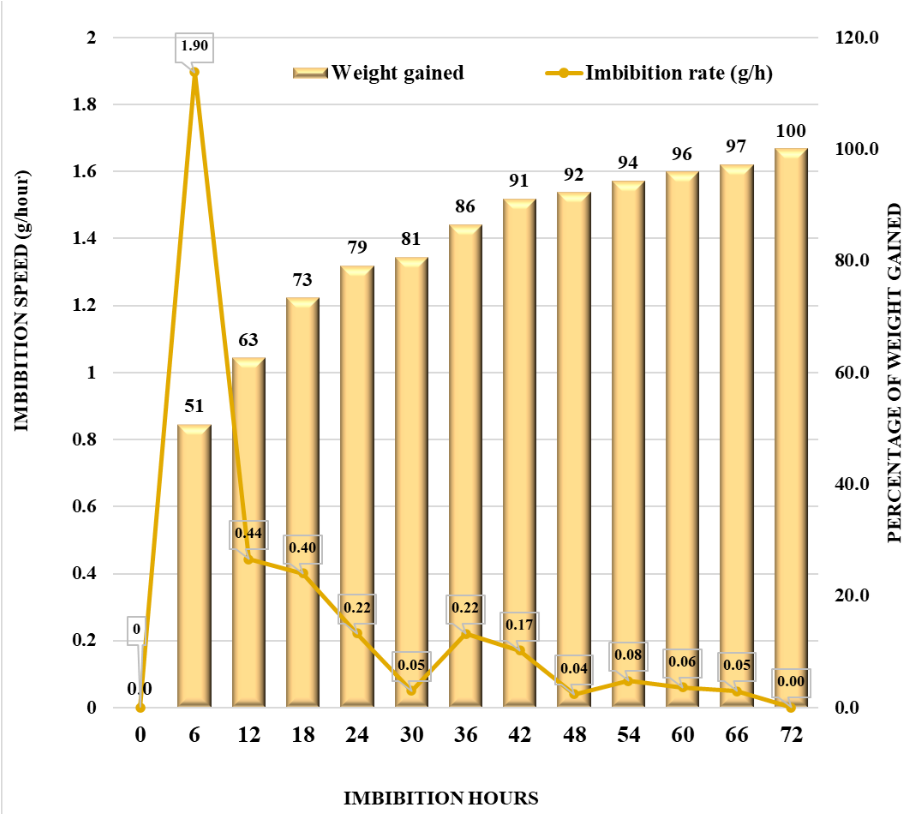

Figure 3. Percentage of fresh weight gained and imbibition rate of freshly collected chincuya seeds exposed to open air for 72 hours and imbibed in distilled water at different pH levels.

In chincuya seeds, water absorption was prolonged and fast in the first six hours. This short period of water entry probably depends only on the barrier by the testa, which is thick, fibrous (VIDAL-LEZAMA et al., 2023), and permeable, as observed with the loss of moisture, as already mentioned. Once through the testa, the endosperm, which is very hard and lipidic, presents resistance; consequently, the speed of absorption decreases. Experiences with anonas seeds indicate that, in general, they are permeable to water, but their absorption is prolonged. In the case of atemoya, the change from Phase I to II occurred between 27 and 34 hours (FERREIRA et al., 2006), reaching Phase III after 234 hours. In the same species, Braga et al. (2010) consider that 36 hours correspond to Phase I. Ferreira et al. (2014) established the end of Phase I water acquisition by determining the asymptotic point of deceleration. They found that seeds of *A. purpurea*, with 16.54 % initial moisture, reached stability at 70 hours and considered that Phase I is completed when a seed moisture content of approximately 31 % is achieved regardless of the time required to reach it. Tables 3 and 4 show that irrespective of the pH of the treatments, the imbibition water is acidic.

Table 3. Final pH of the imbibition water of chincuya seeds immersed for 84 hours in distilled water, with different degrees of acidity.

pH TREATMENT	FINAL pH	
	WET SEEDS	DRIED SEEDS FOR 72 HOURS
5	5.03[z] NS	3.91 NS
7	5.52	4.19
9	5.96	4.21
CV (%)	0.098	0.041

[z] NS. Means without statistical difference according to Tukey's test (p ≤ 0.05).

Table 4. pH dynamics of imbibition treatments of chincuya seeds freshly collected and imbibed for 72 hours.

pH Treatment	Initial pH	Imbibition Hours					
		12	24	36	48	60	72
5	5	5.9	5.2	5	4.9	5.3	4.3
7	7	6	5.5	5.5	5	5.5	5.2
9	9	6.1	5.9	5.4	5.3	5.7	4.9
Gibberellic acid	3.9	4.9	4.7	4.4	4.6	4.3	4.3
Control	6.1	5.9	5.4	5.7	5.6	5.5	5.5

[z] Average of 10 repetitions of 20 seeds each

The recorded pH values indicate the beginning of germination as a respiratory metabolism, where the generation of H^+ and its transport to the cytoplasm are part of this metabolism. The electron transport chain forms a proton gradient across the inner mitochondrial membrane for ATP synthesis (AZCÓN-BIETO; TALÓN, 2000).

Another possibility is that it is the manifestation of the proper metabolism of degradation of lipid reserves since, during the germination process of seeds with high fatty acid content, β-oxidation will generate acetyl CoA that will be used in the glyoxylate cycle. In the glyoxysomes, malic acid is produced to be transferred to the cytoplasm, generating oxaloacetic acid and then sucrose. According to Weitbrecht et al. (2011), in Phase I, several processes coincide with activation of respiration, mitochondrial repair, activation of amino acid metabolism, *de novo* mRNA synthesis and translation, and loss of solutes. These processes also contribute to the variations between the initial and final pH. Also, free radicals produced in highly metabolic cells contribute to this acid metabolism. Gimenez et al. (2017) observed lipid peroxidation during imbibition in *A. emarginata* and Logan et al. (2001) in maize.

Germination of chincuya seeds from the first year of the evaluation was null with any of the treatments tested. Two explanations can be given: a) the seeds were low in gibberellins (GÓMEZ-CASTAÑEDA et al., 2003), and even at pH 7, activation or *de novo* synthesis was not induced, which implies a lack of physiological maturation in the seed (BASKIN; BASKIN, 2014); or b) fermentation could have occurred in the seeds, due to the prolonged imbibition period, as it is known that one of the external factors affecting imbibition is oxygen (MANZ et al*.,* 2005), and it was likely scarce causing the change from aerobic respiration to the fermentation pathway. In lactic fermentation, lactate dehydrogenase uses NADH to reduce pyruvate to lactate and regenerate NAD^+. The same occurs in alcoholic fermentation, where the enzyme alcohol dehydrogenase uses NADH to reduce acetaldehyde to ethanol and regenerate NAD^+. Accumulation of lactate causes a decrease in cytoplasmic pH, and when the cytoplasm is acidified, protein synthesis is suppressed, glycolysis is stopped, and extreme energy deficit occurs (KULICHIKHIN et al., 2008).

Effect on seed germination dynamics. Figure 4 shows the irregular germination behavior of fresh and wet seeds of chincuya, a result of the variability of the undomesticated species. The induction effect of gibberellic acid is observed; however, there was no marked improvement in germination percentage (Figure 5) (the control germinated one percent, one day earlier). This behavior was pointed out in *A. purpurea* by Vidal et al. (2008) and Ferreira et al. (2019), achieving only 29 % in freshly collected seeds. Debeajoun et al. (2000) point out the two mechanisms of action that explain the role of endogenous gibberellins in controlling germination in *Arabidopsis*. The first is the induction of the expression of genes coding for hydrolytic enzymes in the endosperm, as this tissue mechanically restricts root protrusion. Second, it stimulates embryo growth potential, which the ABA produced in the embryo slows. ABA suppresses gibberellin biogenesis, while gibberellins also negatively regulate ABA biogenesis during germination. Therefore, ABA and gibberellins may interact as cause and effect during this process (SHU et al., 2016).

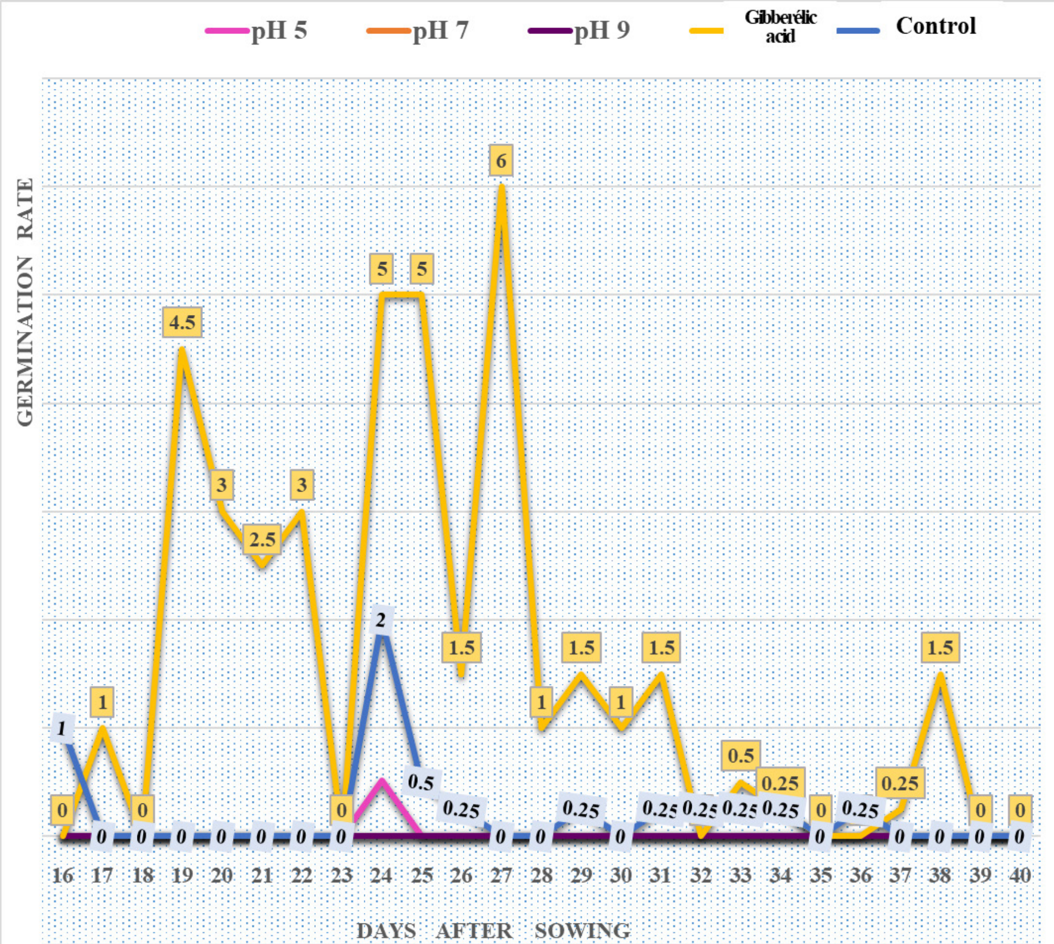

Figure 4. Kinetics of the average daily germination of freshly collected seeds of chincuya, by effect of treatment with distilled water at different pH levels and gibberellic acid.

The slow and progressive increase in germination has been reported in several anona species (VIDAL-LEZAMA et al., 2015b; FERREIRA et al., 2019). The final germination percentage (Table 5; Figures 4 and 5) obtained with this regulator was still low, although it was the highest among all treatments. Morphological dormancy (VIDAL-LEZAMA et al., 2023) conditioned the observed response, given that the seeds were freshly collected. The pH change to the imbibition water did not promote germination; additionally, the values of rotted seeds were high, even with gibberellic acid. One factor that could have caused rotting is the possible decrease in available oxygen promoted by insufficient aeration time (approx. 10 min, every six h) during sample weighting and pH recording of the imbibition water. The negative effect of anoxia or hypoxia on germination has been documented (RAY et al., 2016), and the advantage of water oxygenation (VIDAL-LEZAMA et al., 2018).

Table 5. Effect of pH, gibberellic acid, and distilled water on germination of freshly collected chincuya seeds.

pH Treatment	Germinated Seeds (%)	Dormant Seeds (%)	Dead Seeds (%)
5	0.50 B	60.00 A	31.50 B
7	0.00 B	10.00 B	90.00 A
9	0.00 B	17.89 B	81.05 A
Gibberellic acid	43.16 A	16.84 B	42.63 B
Control	5.79 A	4.74 B	85.26 A
CV (%)	1.64	0.90	0.22

[z] Means with the same letter in columns are significantly equal according to Tukey's test ($p \leq 0.05$).

Vidal et al. (2008), when testing 250, 300, and 350 mg L^{-1} of gibberellic acid and 24- and 48-hour imbibition on chincuya seeds stored for 9.5 months, found that imbibing the seeds for 24 hours in 250 mg L^{-1} of gibberellic acid caused 71 % of final germination. Our germination results of 43 % differ significantly from those reports. Ferreira (2011) also tested gibberellins at 500 mg L^{-1} and increased germination to the highest percentage.

Conclusions

The chincuya seeds contain a high percentage of initial moisture (37%), which is lost rapidly and in high proportion after 24 hours of exposure to the open air. The pH of the imbibition water (5, 7, and 9) did not favor germination. Imbibing seeds in gibberellic acid at 350 mg L^{-1} significantly increased germination to 43 %. The speed of imbibition is high in seeds exposed for three days in the open air, in the first 6 hours, and independent of the pH of the water.

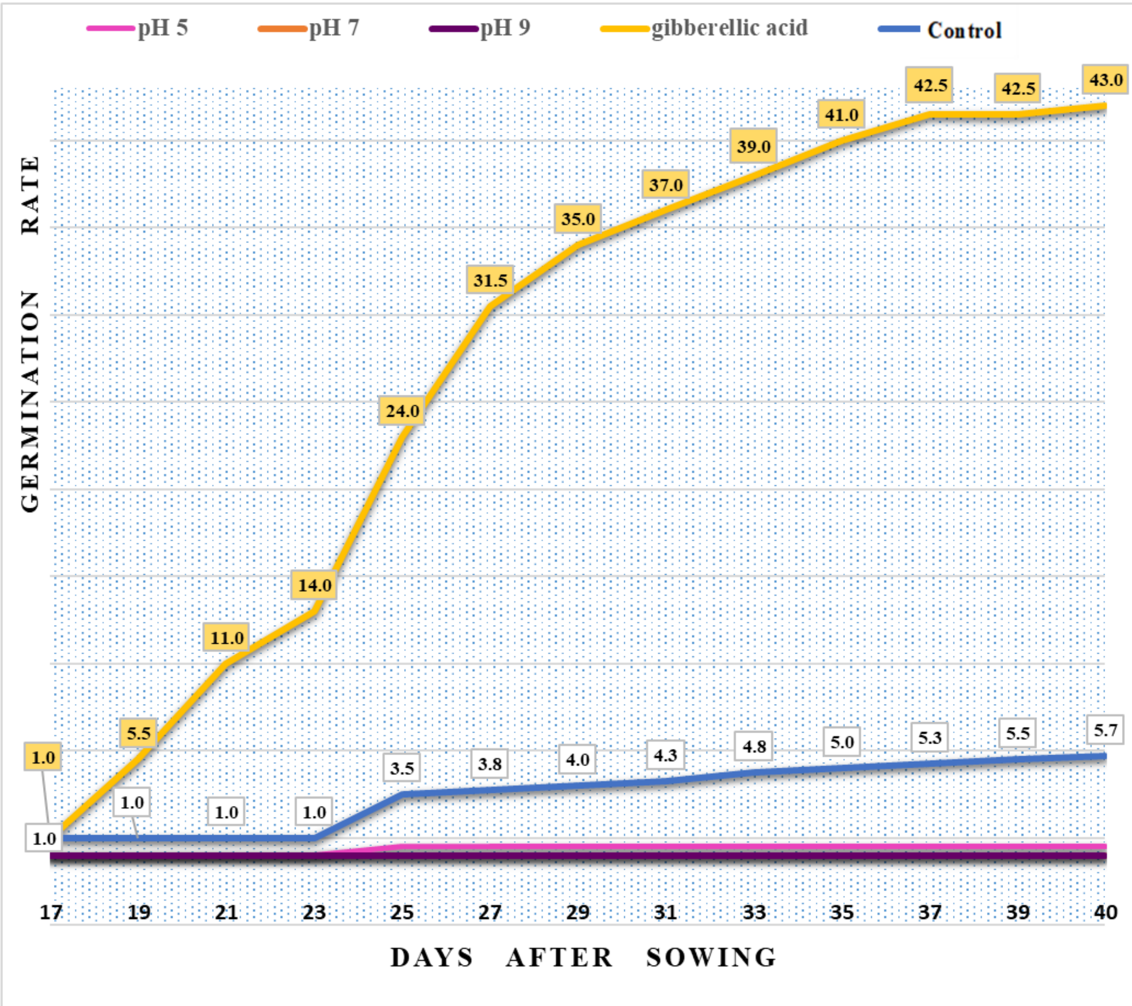

Figure 5. Kinetics of cumulative germination of freshly collected seeds of chincuya, by effect of treatments with distilled water at different pH levels and gibberellic acid.

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