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Delinting and neutralizers residue effect on stored cotton seeds physiological quality determined by phenotyping image analysis

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ABSTRACT: The cottonseed delinting removes lint from the seed coat to improve plantability and maintain the seed's physiological quality, therefore, this work aimed to determine the effect of delinting and neutralizers' residual on the physiological quality of stored cottonseed by using image analysis phenotyping. The experiment was a randomized factorial design of 3 x 4 x 2 and two periods of storage evaluation (0 and 180 days). The cotton seeds were delinted at three different times (2, 4, and 10 minutes), neutralized using four distinct neutralizers (Quicklime, Hydrated lime, Filler lime, and NaOH), and there were seeds with and without neutralizer residue. The evaluations carried out were: water content, seed residual lint imaging, germination, seedling emergence, and seedling length imaging. The neutralizers Quicklime, Hydrated lime, and Filler lime do not need removal from the cotton seed coat. In storage, only the neutralizer NaOH has a high latent effect in lowering cottonseed quality, and the other neutralizers have lower physiological quality when the residue is removed. The image analysis phenotyping is efficient in identifying seedlings' vigor.

Index terms: *Gossypium* L., imaging, lint, neutralization, vigor.

RESUMO: O deslintamento das sementes de algodão remove o linter do tegumento para melhorar a plantabilidade e manter a qualidade fisiológica da semente, por isso, este trabalho teve como objetivo determinar o efeito do deslintamento e do resíduo de neutralizantes na qualidade fisiológica de sementes de algodão armazenado por meio de fenotipagem usando análise de imagens. O experimento foi um delineamento casualizado em fatorial 3 x 4 x 2 e dois períodos de avaliação de armazenamento (0 e 180 dias). As sementes de algodão foram deslintadas em três tempos diferentes (2, 4 e 10 minutos), neutralizadas com quatro neutralizantes (Cal virgem, Calcário hidratada, Calcário filler e NaOH), e utilizou-se sementes com e sem resíduo de neutralizante. As avaliações realizadas foram: teor de água, imagem de linter residual em sementes, germinação, emergência de plântulas e imagem de comprimento de plântulas. Os neutralizantes Cal virgem, Calcário hidratado e Calcário filler não precisam ser removidos do tegumento da semente de algodão. No armazenamento, apenas o neutralizante NaOH apresenta alto efeito latente na redução da qualidade da semente de algodão, e os demais neutralizantes apresentam inferior qualidade fisiológica quando o resíduo é removido. A fenotipagem por análise de imagem é eficiente na identificação do vigor das plântulas.

Termos para indexação: *Gossypium* L., análise de imagem, línter, neutralização, vigor.

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INTRODUCTION

Cotton production faces many challenges in field management that increase risk and causes limitation in economic productivity (Snider et al., 2016). One of the strategies to overcome some of these challenges is to use high-quality cottonseed that guarantees a uniform stand establishment leading to high yields (Afzal et al., 2020). Thus, to maintain the cottonseed's high performance after harvest, several processing steps need attention to avoid losses in physiological seed quality.

The first step to obtaining standardized cotton seeds is removing the fiber in a ginning process, although a short length of lint remains on the seed coat. Delinting methods are the next step to remove the remaining lint; and the sulfuric acid followed by neutralization is chosen by most companies and small producers (Queiroga and Mata, 2018; Dowd et al., 2019; Maeda et al., 2021). Many researchers have refined the use of sulfuric acid (Ferraz et al., 1977; Biradarpatil and Macha, 2009; Dowd et al., 2019); however, the neutralization has not been given proper attention.

The neutralization is applied on cottonseeds to neutralize sulfuric acid residues and avoid problems in seed quality, storage, and the environment. There are different neutralizers, such as sodium, potassium, calcium, and magnesium hydroxides, known as stress stabilizers (Sallum et al., 2010), and variations of limestone. After neutralization time, the neutralizer residue can be washed with water or not before drying the cotton seeds to their ideal moisture content.

The neutralizer residue on cotton seed coat has been considered a question about its effect on the physiological quality during storage. Most research in the literature focuses on the sulfuric acid outcome on cottonseed quality like in (Queiroga and Mata, 2018) and (Maeda et al., 2021), and what would be best in terms of removing the neutralizer after neutralization or maintaining it is unclear. In addition, knowing which neutralizer could be applicable for these two options is another piece of information to consider, especially under natural storage conditions.

Furthermore, cottonseed passes through a long period of processing until reaches the market; this process must be accurate to maintain all genetic, physical, physiological, and health attributes necessary to guarantee high-quality seeds (Santos et al., 2015). Likewise, image analysis could be a helpful tool to verify cottonseed processing success since; it has been demonstrated to phenotype various morphological characteristics with accuracy in plant tissues, such as leaves (Aboukarima et al., 2017) and seeds and seedlings (Lima et al., 2018a; Lima et al., 2018b). The phenotyping imaging analysis could help identify if neutralizer residue affects seedling vigor, particularly when associated with delinting times.

Therefore, investigating how neutralizer residue interacts with stored cottonseed is necessary to improve the delinting chain by helping processing companies and small producers in their decision-making. Here we address the hypothesis that different neutralizers could affect cottonseed physiological quality differently when there is residue or not on the seed coat and seeds are stored at room temperature. This work aimed to determine the effect of delinting and neutralizers' residual on the physiological quality of stored cottonseed by using image analysis phenotyping.

MATERIAL AND METHODS

Plant material and experimental design

The experiment was carried out at the *Universidade Federal de Lavras* from December 2020 to May 2021 using lint cotton seeds cultivar DP1746B2RF, the initial moisture content of the seed lot was 8%. The experiment was carried out in a completely randomized design factorial of 3 x 2 x 4 (delinting time x neutralizer residue x neutralizers) with two storage periods (0 and 180 days) and four replications. The storage environment was at room temperature (± 25 °C).

Delinting, Neutralization and sample standardization

The seed lot was homogenized, divided into three samples, and submitted at different times of delinting (2, 4, and 10 minutes). The delinting process was done manually with sulfuric acid (98%) in a proportion of 1 L per 7 kg of lint seed. After the delinting, the sulfuric acid excess was removed by washing the seeds in a 2 L becker containing water stirred for one minute. The seeds were divided into four subsamples at each time of delinting and placed into four beckers (2 L) containing one type of neutralizer each (Quicklime, Hydrated lime, Filler lime, and Sodium Hydroxide). The concentration of all neutralizers was 10% (10 g per 100 mL of water) and stirred for one minute (the liquid volume used was enough to cover the seeds). After neutralization, subsamples from each time of delinting and each neutralizer were divided again into two subsamples, where one subsample was washed in running water to remove all neutralizer residue (two minutes), and the other subsample was drained, keeping the neutralizer residue on the seed coat. The seeds were dried on an air-circulated stove at 30 °C for 24 hours. The neutralizers pH was checked, and all limes presented pH 10.0 and NaOH pH 14.0. Also, the seeds pH was checked, showing pH 7.0 for all limes and pH 10.0 for NaOH. The seeds pH measurement was made using 50 seeds of each treatment submerged in 75 mL of distilled water for 30 minutes, and pH analyzed with a pH meter equipment.

The neutralizers used were industrial products: Quicklime (CaO) - calcination of $CaCO_3$; Hydrated lime ($Ca(OH)_2$) - hydration of CaO; Filler lime ($Ca(OH)_2$) - fine grind PRNT of 134%; and Sodium Hydroxide PA (NaOH) – pure 100%.

After dried, the cotton seeds were classified using oblong sieves (10 x 5 mm) and packed in multiwall paper bags. During packing, all treatments were divided into two paper bags for storage (0 and 180 days).

Evaluated traits

Water content: oven method at 105 °C ± 3 °C for 24 hours, using two replications of 10 g seeds (Brasil, 2009); results were expressed in percentages.

Seed residual lint imaging: to quantify the amount of lint residue left on the seed coat, image analysis was made using the Groundeye® equipment/software from Tbit©. One side of 100 seeds per replication was used to take the images, and only cotton seeds without neutralizer residue had image captured for accuracy in the quantification of lint left on the seed coat. The quantification happened by capturing the seed color, which had a color variation for delinted tegument (brown color) and remaining lint (yellow color) around the seed. The average percentages of yellow color from the 100 seeds were quantified, and the results were expressed in percentages of residual lint. Also, to compare seeds with and without neutralizer residue from each time of delinting images were captured.

Germination: 50 seeds per replication were sown on a paper towel moistened with 2.5 times the weight of the paper in water and incubated in a chamber at 25 °C; the results were expressed in the percentage of normal seedlings evaluated 12 days after sowing (Brasil, 2009).

Seedling emergence: substrate was composed of sand and soil in a 2:1 ratio and placed in plastic containers; after sowing, 50 seeds per replication, the containers were incubated at a controlled room temperature of 25 °C. The results were expressed as a percentage of emerged seedlings 14 days after sowing.

Seedling length imaging: four replications of 20 seeds were sown on a towel paper, moistened for the germination analysis, and incubated in a chamber at 25 °C for five days. The seeds were laid on the paper mismatched to avoid overlap of the seedlings. The Groundeye® equipment/software was used to capture the seedlings' images and measure the hypocotyl and radicle lengths. The results were expressed in centimeters. Pictures of the seedlings of each treatment were taken to create one image with all treatments combined.

Statistical analysis

The results of germination, seedling emergence, seed residual lint, and seedling were submitted to statistical analysis of variance using the F test with the comparison of means by the Scott-Knott test ($p < 0.05$). The water content results were expressed in average. The software used for statistical analysis was SISVAR (Ferreira, 2014), and the graphics were designed on Sigmaplot 10.0 and images on PowerPoint 2016.

RESULTS AND DISCUSSION

The results from delinting the seeds at three different times (2, 4, and 10 minutes) followed by neutralization with different neutralizers (Quicklime, Hydrated lime, Filler lime, and NaOH), and the presence or not of the residue from the seed coat showed a significant interaction between the treatments. The water content measured to verify the amount of moisture content cotton seeds absorbed from the initial 8% showed an average between treatments of 12% (0 days) and 8% (180 days), confirming the efficiency of the drying method and storage. In storage conditions, seed quality can deteriorate as temperature and relative humidity varies over time. Maintaining seed moisture content at low levels is determinant to slower the natural aging process (Shaban, 2013).

The seed residual lint imaging evaluation results showed significant differences between delinting times (Figure 1). The 2 minutes of delinting had an average of 49% of lint around the seed coat, 4 minutes 29%, and 10 minutes 11%. Interestingly, the 2 min delinting removed ~50% of the lint from the seeds, and when the time was doubled, there was a decrease in delinting fastness, where only ~70% of lint was removed at 4 min and ~90% at 10 min. To increase the delinting efficiency, the ratio between sulfuric acid volume and cottonseed should be changed to accelerate the delinting process.

The amount of lint on the seed coat determines the appropriate ratio of sulfuric acid to cottonseed for delinting. Since cotton fibers are high in cellulose content (Morandim-Giannetti et al., 2012), and sulfuric acid makes dehydration reactions where the water released catalyzes these reactions by hydrolyzing the cellulose chains (Teixeira et al., 2010), the speed of these reactions is influenced by the cellulose content during delinting, which varies among cotton genotypes and delinting methods. Therefore, there is no universal time and sulfuric acid ratio for cotton delinting, and it is necessary to test the cotton genotype and delinting method to ensure successful lint removal without damaging seed quality. When the physiological quality of the cottonseed was evaluated to verify this relationship between sulfuric

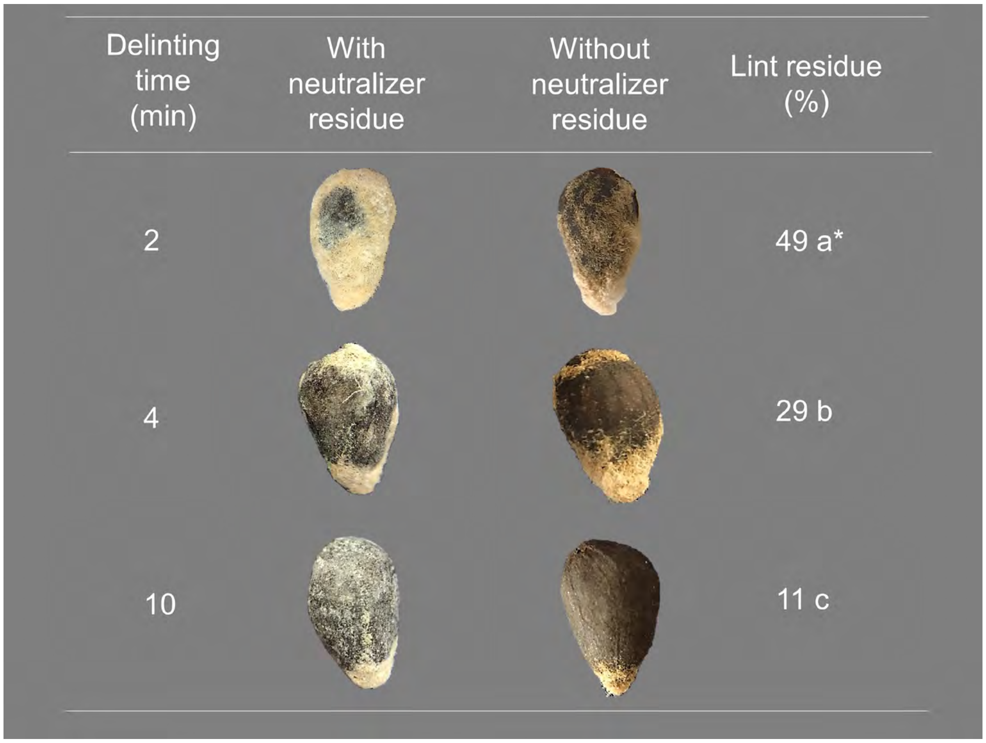

Figure 1. Cotton seed residual lint imaging (%) after delinting (2, 4, and 10 minutes) and neutralization (with and without neutralizer residue). *Means followed by distinct letters in the column differ by the Scott-Knott test at $p < 0.05$. Coefficient of variation: 14.90%.

acid and neutralization in germination analysis, results showed no differences (0 days) between the treatments with or without neutralizers' residue in most delinting times (Figure 2A). The only differences found were in the neutralizer Hydrated lime in 4 min, where seeds without the neutralizer residue decreased germination by 9% compared to seeds with residue; Filler lime showed inverse results at 10 min whereas, seeds with residue presented a decrease of 9% in germination compared to seeds without the residue.

Likewise, the neutralizer NaOH was the only one with germination reduction in all times of delinting, showing a decrease in germination in seeds with the residue. It was verified a reduction of 93% (2 min), 85% (4 min), and 100% (10 min) in germination compared to seeds without residue in this same neutralizer. The toxicity during germination has been reported in soybean seeds due to chemical treatments; the concentration of these products in contact with the seed is 3.500 times more compared to the emergence in soil (Tunes et al., 2020). Although the neutralizer residue significantly affected germination, its removal proved to be beneficial, resulting in germination rates of approximately 80% across all delinting times.

At 180 days of storage, germination decreased as a result of normal aging in all treatments, however the cotton seeds with NaOH residue were highly affected by the residue and germination was below ~5%, showing a high number of abnormal seedlings during the evaluation (Figure 2B). This could be the toxicity effect of the NaOH during storage added to its high residual concentration on the seed during germination.

The seeds without the NaOH residue (180 days) showed that the 2 min treatment maintained seed germination (76%), similar to the other neutralizers in the same condition. Also, it was observed a higher loss of germination at 4 min (~20%) and 10 min (~30%) compared to other neutralizers at each time of delinting. Furthermore, it was verified that the increasing time of delinting had progressive germination loss. This tendency might be due to the sulfuric acid abrasion, making it easier for neutralizer penetration inside the tegument during neutralization, which could not be removed by the washing process. Soliman and Abbas (2013) have shown the abrasion of sulfuric acid use to overcome physical dormancy in *Cassia fistula* L. seeds, which proves the capacity of this chemical to peel tegument tissue allowing water to come through.

The seedling emergence analysis at 0 days followed the same tendency as the germination with the neutralizers Quicklime, Hydrated lime, and Filler lime, as seeds were better performed after the neutralization (Figure 3A). Between these neutralizers, the treatments that showed a decrease in seedling emergence, were the ones without the residue Hydrated lime (13%), and Filler lime (5%) at 2 min, and Quicklime at 4 (7%), and 10 (5%) min of delinting. The seedling emergence results of seeds with NaOH residue were higher than in the germination analysis, which could be explained by more dispersion of the neutralizer around the seed in the soil; the emergence increased to 68% (2 min), 74% (4 min), and 75% (10 min). These results confirm how toxic NaOH could be to the cottonseed physiological quality since emergence was still below other neutralizers' average in the same delinting time. Luo et al. (2018) have confirmed how valuable simple seed test analyses can be to indicate toxicity in the seed germination process.

At 180 days, the seedling emergence results (Figure 3B) showed a decrease in emergence for seeds without the residue of Quicklime (15%), Hydrated lime (8%) at 2 min, and Filler lime (13%) at 4 min compared to the seeds with their residue. And Hydrated lime and Filler lime performed the best seed emergence in 10 min regardless of residue presence. The cotton seeds with NaOH residue showed a decrease of emergence in 42% (2 min), 25% (4 min), and 30% (10 min) compared to the same treatments in 0 days. These results suggest that the neutralizer residue can have a latent toxicity during seed storage, indicating that it is best to remove the NaOH residue before sowing or to shorten the delinting time to around two minutes to minimize abrasion to the tegument and limit the absorption of NaOH. Although NaOH is commonly used in tissue culture to adjust the pH of artificial media, high concentrations can cause salt stress (Na^+) in plant tissue (Doungous et al., 2022), which may explain the poor performance of cotton seeds, especially when there is residue. The hypocotyl measurements in 0 days (Figure 4A) showed no significant differences between seedlings from seeds with or without the residue of Quicklime, Hydrated lime, and Filler lime; the hypocotyl average size was above 4.0 cm. The cottonseed with the NaOH residue presented a significant difference in size; the hypocotyl was below 2 cm at 2 min and 4 min, indicating an abnormality of the seedlings and no germination in 10 min. However, seedlings from seeds without NaOH residue, besides bigger hypocotyl size,

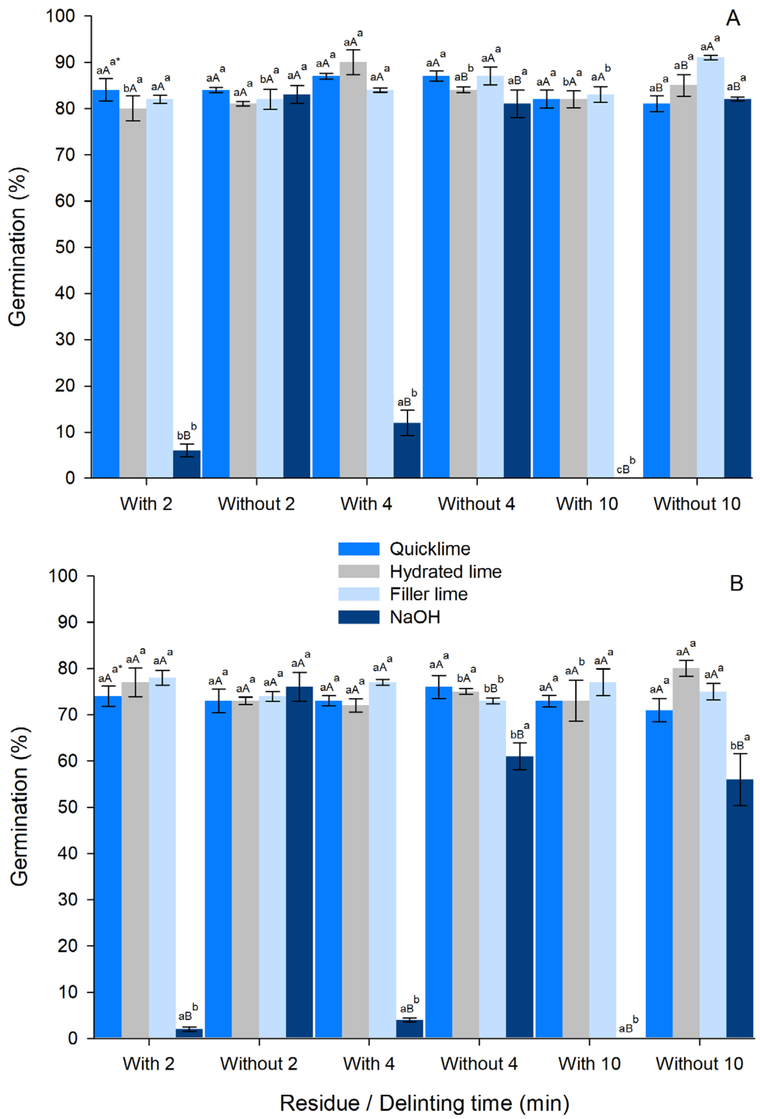

Figure 2. Germination (%) of cottonseed submitted to different delinting times (2, 4, and 10 min), neutralizers (Quicklime, Hydrated lime, Filler lime, and NaOH), neutralizer residue (with and without), and the storage period 0 (A) and 180 days (B). *Means followed by distinct lowercase letters differ in the same neutralizer residue and neutralizer between different delinting times; uppercase letters differ in the same neutralizer residue and delinting time between different neutralizers, and superscript letters differ in the same delinting time and neutralizer between neutralizer residue (Scott-Knott test at $p < 0.05$). Coefficient of variation: 4.55% (A) and 7.13% (B).

showed that when the time of delinting was increased, the hypocotyl was significantly reduced from 4.31 cm (2 min) to 3.77 cm (4 min) and 2.91 cm (10 min).

Seeds stored at 180 days (Figure 4B) showed hypocotyl size reduction in all treatments around 1.0 cm, except for NaOH, and there were no differences between seeds with or without residue for these neutralizers (Quicklime, Hydrated lime, and Filler lime). Seeds from NaOH with residue treatment highly decreased hypocotyl size after storage, where less than 1.0 cm was measured at 2 min and 4 min, and zero cm at 10 min. Seedlings from NaOH without residue treatment presented no difference in size compared to other neutralizers at 2 min (3.44 cm), and at 4 (3.24 cm) and 10 min (2.59 cm), they were inferior, which could be the effect of the sulfuric abrasion and neutralizer referred previously.

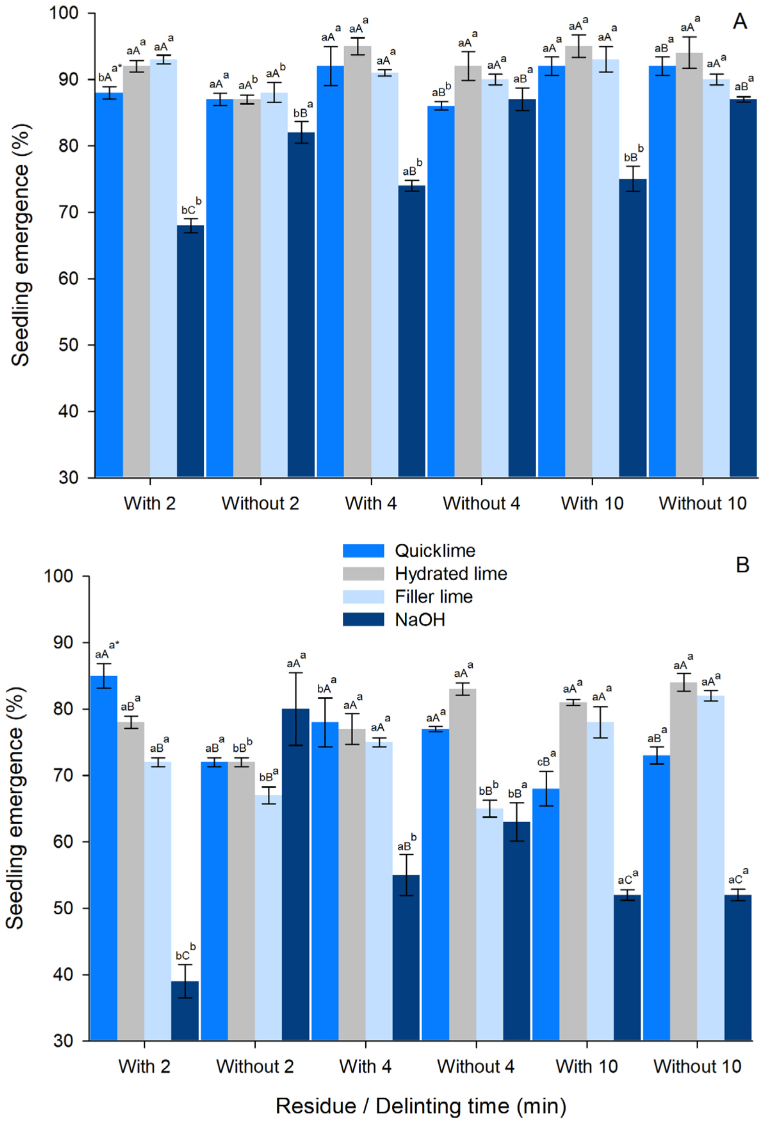

Figure 3. Seedling emergence (%) of cottonseed submitted to different delinting times (2, 4 and 10 min), neutralizers (Quicklime, Hydrated lime, Filler lime and NaOH), neutralizer residue (with and without), and the storage period 0 (A) and 180 days (B). *Means followed by distinct lowercase letters differ in the same neutralizer residue and neutralizer between different delinting times; uppercase letters differ in the same neutralizer residue and delinting time between different neutralizers, and superscript letters differ in the same delinting time and neutralizer between neutralizer residue (Scott-Knott test at $p < 0.05$). Coefficient of variation: 3.19% (A) and 6.19% (B).

Measuring isolated parts of the seedlings is a good indicator of how treatments affect tissues reflecting in vigor (Lima et al., 2018b), a well-developed shoot guarantees the efficiency of photosynthesis, and the use of lime neutralizers did not influence hypocotyl growth.

The radicle results in 0 days (Figure 5A) showed no difference in size between the neutralizers Quicklime, Hydrated lime, and Filler lime when residue was present on the seed coat at all delinting times; the seedlings measured around 9.0 cm. However, when residue was removed, there was a significant difference in seeds with 10 min of delinting; the radicle decreased size by ~1.0 cm compared to the ones with the residue, reflecting 13% of the loss.

Seedlings measured from NaOH with residue treatment were inferior in size compared to the same neutralizer

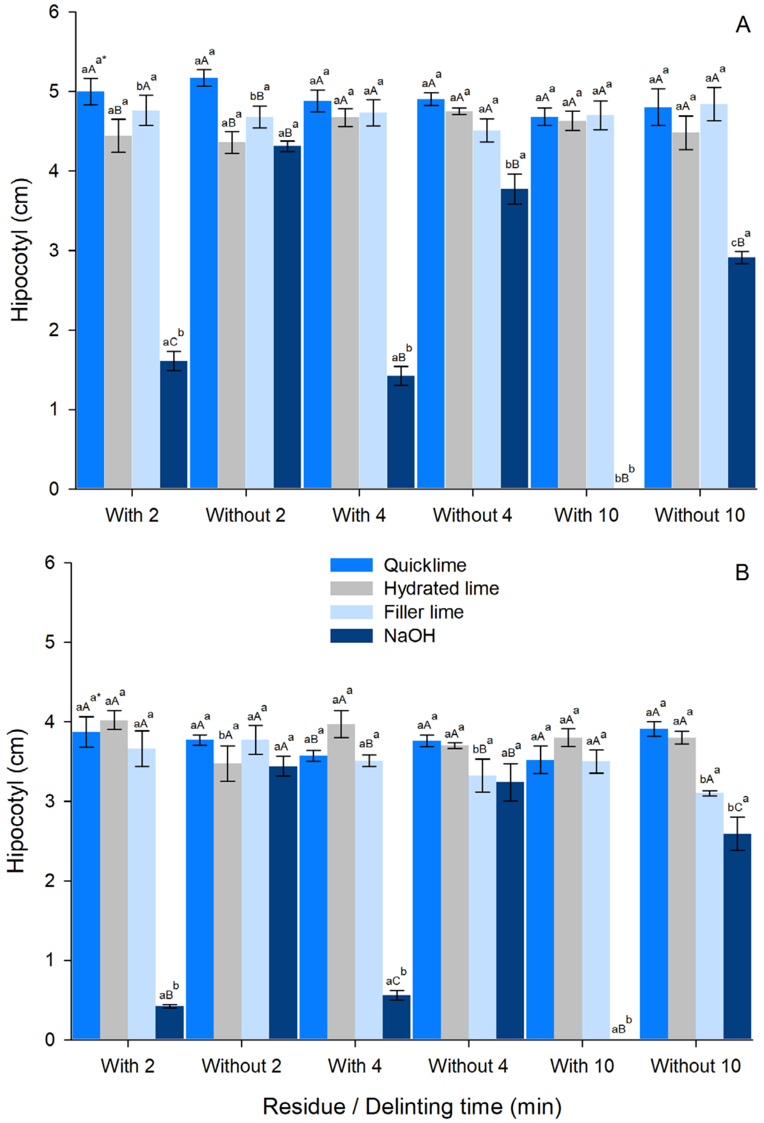

Figure 4. Hypocotyl imaging (cm) of cottonseed submitted to different delinting times (2, 4 and 10 min), neutralizers (Quicklime, Hydrated lime, Filler lime and NaOH), neutralizer residue (with and without), and the storage period 0 (A) and 180 days (B). *Means followed by distinct lowercase letters differ in the same neutralizer residue and neutralizer between different delinting times; uppercase letters differ in the same neutralizer residue and delinting time between different neutralizers, and superscript letters differ in the same delinting time and neutralizer between neutralizer residue (Scott-Knott test at $p < 0.05$). Coefficient of variation: 7.15% (A) and 8.89% (B).

without the residue at all times of delinting. Furthermore, seedling size decreased following the increase in delinting time, measuring 2.43 cm (2 min), 1.85 cm (4 min), and 0.00 cm (10 min). The same effect happened with seedlings from the NaOH without the residue treatment measuring 9.04 cm (2 min), 5.89 cm (4 min), and 4.93 cm (10 min); the comparison between NaOH with and without residue showed 74%, 69%, and 100% of loss, respectively. Only seedlings of NaOH without residue at 2 min showed a similar radicle size compared to the other neutralizers. Seedlings are sensitive to environmental stresses such as the presence of chemicals around them that disturbs their potential growth (Zhang et al., 2020); NaOH has shown an effect in shoot and radicle growth in this study, mainly when residue is present.

Curiously, the radicle size at 180 days (Figure 5B) decreased when the residue was removed from the seeds for the neutralizers Quicklime, Hydrated lime, and Filler lime. The losses of radicle size at 2 min were 19%, 20% , and 17%, at 4 min 25%, 18% and 2.5%, and at 10 min 16%, 30%, and 13%, respectively. hese treatments decreased approximately 2.0 cm compared to the radicle from the same neutralizers with residue in all times of delinting. It could be highlighted that the reduction in radicle size indicates the effect of adding one more washing step to the delinting process, since it lowers seed quality, particularly in terms of radicle growth. The importance of analyzing seedlings in various tissues to identify vigor-related issues, as previously noted by Zhang et al. (2020) and Lima et al. (2018b) showed how some conventional seed test could obscure these results.

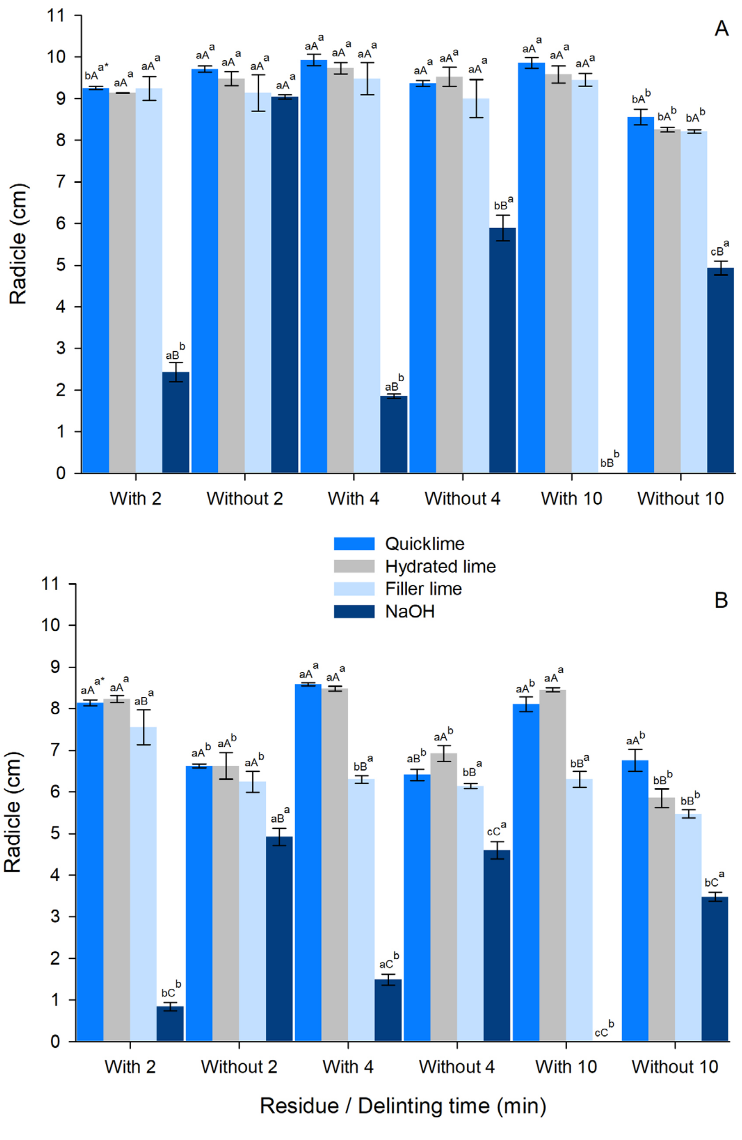

Figure 5. Radicle imaging (cm) of cottonseed submitted to different delinting times (2, 4 and 10 min), neutralizers (Quicklime, Hydrated lime, Filler lime and NaOH), neutralizer residue (with and without), and the storage period 0 (A) and 180 days (B). *Means followed by distinct lowercase letters differ in the same neutralizer residue and neutralizer between different delinting times; uppercase letters differ in the same neutralizer residue and delinting time between different neutralizers, and superscript letters differ in the same delinting time and neutralizer between neutralizer residue (Scott-Knott test at $p < 0.05$). Coefficient of variation: 7.59% (A) and 7.56% (B).

Furthermore, the neutralizer Filler lime (Figure 5B) treatment exhibited inferior performance in seedling radicle size compared to Quicklime and Hydrated lime when residue was kept on the seed coat, which makes this neutralizer not appropriate for situations of storage. The NaOH treatments followed the same tendency previously described presenting radicles below 2.0 cm (with residue) and 5.0 cm (without residue) at all times of delinting.

The seedling phenotyping (Figure 6) showed a more detailed image of how seedlings were affected by the neutralizers treatments before and after storage, which gives a better perception of the seed lot. It has been proven that weak seedlings reflect in many problems during plant development, such as sensitivity to insects and diseases and lower productivity. Most seed tests inform an overall result of the seed quality made in bulk or batches, which most times could mask the actual condition of the seed lot (ElMasry et al., 2019). While image analysis phenotyping has been applied to many crops, including beans, corn, and wheat, there is a lack of studies on using this method to assess the physiological quality of cottonseeds. However, our study demonstrated the potential of phenotyping as a valuable tool in the cottonseed delinting chain to quantify lint and determine seed quality.

To summarize, this study has demonstrated the precision and accuracy of equipment that uses digital cameras and software to obtain accurate measurements of seedlings, which can identify differences in vigor that are imperceptible to the human eye in traditional vigor tests. The use of digital scanners and cameras in conjunction with image processing software has proven to be a more objective and reliable method for plant analysis than older, more subjective measuring techniques (Ahmad et al., 2015; Aboukarima et al., 2017).

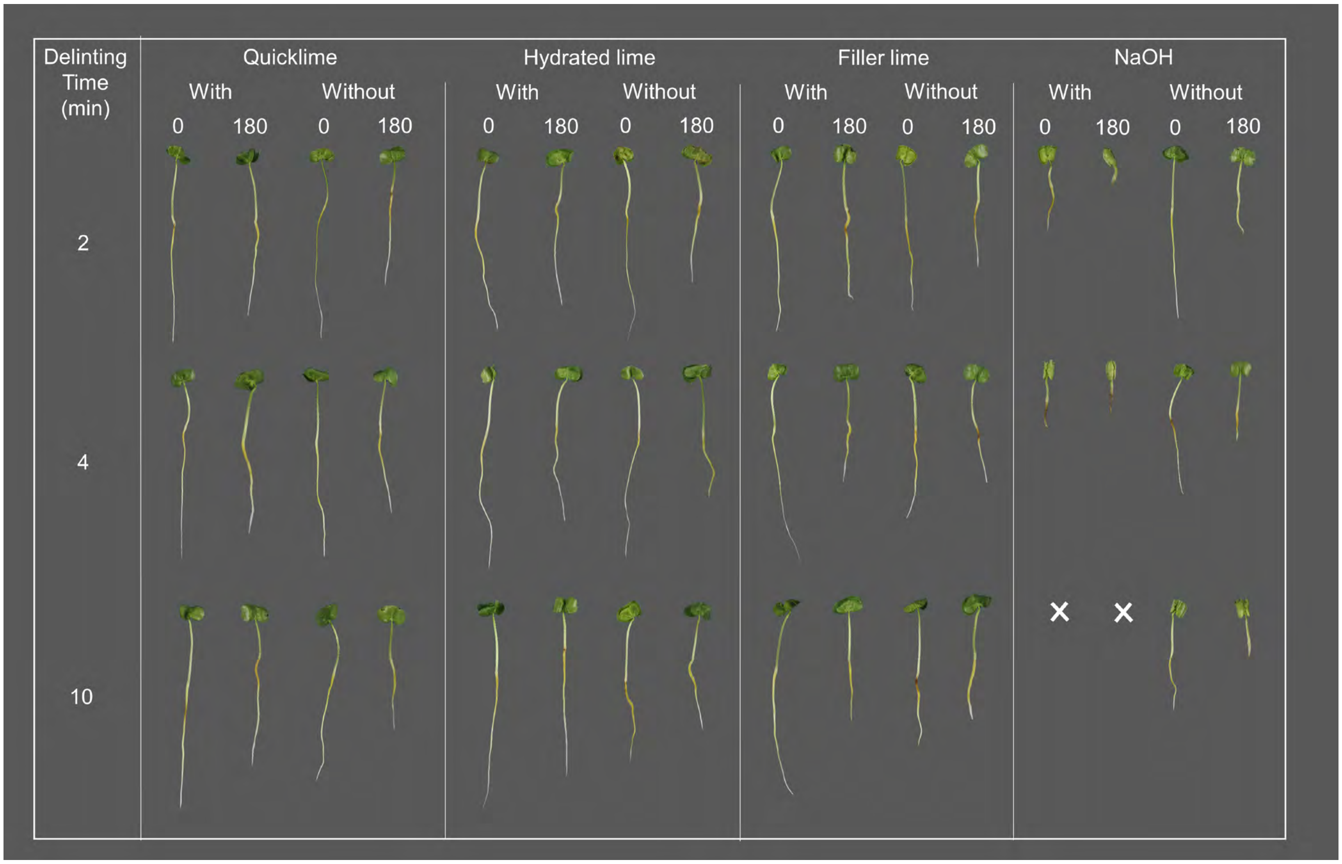

Figure 6. Phenotyping analysis of cottonseed seedling five days of germination after delinting (2, 4, and 10 minutes) and neutralization (with and without neutralizer residual) using different neutralizers (Quicklime, Hydrated lime, Filler lime, and NaOH) during storage 0 and 180 days. Source: Elaborated by the authors.

CONCLUSIONS

Cottonseed quality is not adversely affected by the residue on the seed coat from neutralizers Quicklime, Hydrated lime, and Filler lime.

NaOH has no detrimental effect on cottonseed quality when the residue is removed, and a delinting time should be approximately 2 minutes at 0 days.

Quicklime and Hydrated lime help maintain cottonseed quality for up to 180 days of storage, whereas the use of NaOH can significantly reduce physiological quality, whether or not residue is present.

Removing neutralizer residue from the cottonseed during the delinting process affects cottonseed vigor at 180 days of storage.

Delinting times alone do not have a significant impact on cottonseed physiological quality, but when used in conjunction with NaOH, the negative effects are more pronounced.

Image analysis is an effective means of measuring the amount of remaining lint on cotton seed coats, and can accurately reveal differences in seedling vigor between treatments that may be overlooked by conventional seed quality analysis.

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