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Germination and biochemical changes in 'Formosa' papaya seeds treated with plant hormones

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ABSTRACT. This study aimed to investigate the effects of growth regulators on germination rates and biochemical compound concentrations in *Carica papaya* L. seeds ('Formosa' group). The seeds were harvested from fruits at maturation stages 3 and 5 (50 and 75% yellow fruit skin, respectively). The effects of 2-chloroethylphosphonic acid (CEPA), KNO_3 and gibberellic acid (GA_3) on seed germination, germination index speed, soluble sugars, starch, lipids, soluble proteins and total proteins of the papaya seeds were evaluated. The seeds from stage 5 showed a higher rate of germination 30 days after sowing than did the seeds from stage 3. Treatment with CEPA decreased seed germination, apparently due to decreased starch mobilization; the opposite response was observed following KNO_3 treatment. GA_3, alone or in combination with KNO_3, stimulated an increase in lipid mobilization. In general, with the exception of CEPA, all growth regulators tested were effective in overcoming seed dormancy, and KNO_3 was the most effective. The seeds from stage 3 fruits treated with KNO_3 or KNO_3 + GA_3 had higher rates of germination at 14 days.

Keywords: *Carica papaya* L, CEPA, ethylene, gibberellins, KNO_3, reserve compounds.

Germinação e alterações bioquímicas em sementes de mamão do grupo 'Formosa' tratadas com fitohormônios

RESUMO. O objetivo deste trabalho foi investigar o efeito de reguladores de crescimento na germinação e na variação de alguns compostos em sementes de *Carica papaya* L. grupo "Formosa", extraídas de frutos nos estádios de maturação 3 e 5, correspondendo a 50 e 75% da casca com cor amarela, respectivamente. Avaliaram-se os efeitos do ácido 2-cloroetilfosfônico (CEPA), KNO_3 e GA_3 na germinação, índice de velocidade de germinação, no conteúdo de açúcares solúveis, amido, lipídios, proteínas solúveis e totais das sementes. As sementes-controle obtidas de frutos no estádio 5 apresentaram maior germinação aos 30 dias após a semeadura em relação às do estádio 3. O CEPA promoveu diminuição na germinação das sementes, aparentemente, associado com a redução da mobilização de amido, sendo revertida com KNO_3. O GA_3, isoladamente e em associação com o KNO_3, promoveu maior mobilização de lipídios. Em geral, com exceção do CEPA isoladamente, todos os reguladores de crescimento testados foram eficientes na superação da dormência. O KNO_3 mostrou-se o composto mais eficiente em aumentar a germinação. Sementes do estádio 3 tratadas com KNO_3 ou KNO_3+GA_3 apresentaram maior germinação aos 14 dias.

Palavras-chaves: *Carica papaya* L, CEPA, etileno, giberelina, KNO_3, compostos de reserva.

Introduction

Dormancy can be defined as the inability of viable seeds, even under favorable conditions, to germinate (FINCH-SAVAGE; LEUBNER-METZGER, 2006). Several factors are involved in the regulation of dormancy, including hormones such as abscisic acid (ABA), gibberellins (GAs), ethylene, cytokinins and others (CARRERA et al., 2008; CHANDRA et al., 2007; FINCH-SAVAGE; LEUBNER-METZGER, 2006; HOLDSWORTH et al., 2008; RAMAIH et al., 2003; RIEFLER et al., 2006). In addition, environmental factors such as light and temperature may affect seed dormancy and germination (KONDO et al., 2011; SOCOLOWSKI et al., 2010). The complexity of the hormonal responses and their overlapping functions support the occurrence of intensive cross-talk among the various signaling pathways (RAZEM et al., 2006).

In some plant species, NO_3^- can act as a germination promoter, possibly in association with GAs (GASHI et al., 2012). This effect appears to be independent of the reduction of NO_3^- to nitric oxide (NO), suggesting that NO_3^- may act as a germination trigger (SÁNCHEZ et al., 2010).

Indeed, the accumulation of NO_3^- in soil may overcome seed dormancy by leading to changes in the concentrations of reserve compounds; this effect can be evaluated by analyzing changes in the concentrations of compounds such as sugars, starch, amino acids, fatty acids and others, as proposed by Alboresi et al. (2005). These authors also suggested that NO_3^- accumulation in seeds may be associated with a lower requirement of GAs for germination. NO_3^- can also have an effect on the pentose phosphate pathway, which supplies the energy that is required during the early stages of germination (BETHKE et al., 2006).

Brazil is the largest producer of papaya fruits worldwide, having produced 18 billion kg in 2009, with retail sales of 700 million dollars (AGRIANUAL, 2011). Papaya seeds have physiological dormancy, and their germination process is slow and non-uniform (LOPES; SOUZA, 2008). Such dormancy is believed to be associated with inhibiting compounds (TOKUHISA et al., 2007a) that are believed to act mainly in the sarcotesta (TOKUHISA et al., 2007b).

Despite some attempts to overcome dormancy and/or to accelerate germination, little is known about the physiological mechanisms associated with seed dormancy regulation in papaya (TOKUHISA et al., 2007a and b). Some evidence suggests that "Formosa" papaya seeds display a significant increase in germination after immersion in KNO_3 solutions (TOKUHISA et al., 2007b). GAs have often been employed to break the dormancy of papaya seeds (LOPES et al., 2009; TOKUHISA et al., 2007b). However, possible roles for other growth regulators, such as ethylene, that are associated with seed dormancy in papaya have not been investigated. In this study, we investigated the effects of 2-chloroethylphosphonic acid (CEPA), which is an ethylene-releasing substance, gibberellic acid (GA_3) and KNO_3, applied individually or in combination, on germination and reserve mobilization as a means of increasing the understanding of dormancy regulation in seeds of papaya fruits at two maturity stages.

Material and methods

This study was conducted in Viçosa (20° 45'S, 42° 15' W), Minas Gerais State, Brazil, with seeds of 'Formosa' papaya (*Carica papaya* L.). Seeds were harvested from hermaphrodite fruits at maturity stages 3 ('half mature', 50% yellow skin) and 5 (mature, more than 75% yellow skin), as described by Aroucha et al. (2005) and Lopes et al. (2009). These seeds are hereafter referred to as seeds of stages 3 and 5, respectively. After harvest, the seeds were rubbed between sheets of paper and washed with running tap water to remove their sarcotesta. They were then left on paper towels to dry out under laboratory conditions (± 20°C) until reaching approximately 10% moisture content, which occurred within approximately 10 days. The water content of the seeds was determined using four replicates of 50 seeds following drying in an oven at 105 ± 3°C for 24 hours (BRASIL, 2009).

Experiment 1

Four replicates of 50 seeds at each maturation stage were germinated in rolls of germitest paper towel moistened with a CEPA solution equivalent to 2.5 times the mass of dry paper. The CEPA concentrations used were 0, 5 x 10^{-6}, 1 x 10^{-5}, 1 x 10^{-4}, 5 x 10^{-4}, and 1 x 10^{-3} mol dm^{-3}. The paper towel rolls were kept in a Mangelsdorff germinator (Tecnal, Piracicaba, Brazil) under alternating temperatures of 20-30°C (16 hours dark/8 hours light, respectively) for the germination tests. Germination was monitored for 30 days; seedlings were considered normal if they were at least 5 cm long. Germination percentages were counted at 14 and 30 days after sowing (BRASIL, 2009).

Experiment 2

The seeds were treated as in Experiment 1. Before being wrapped in moistened paper towel rolls, the seeds were immersed in solutions of KNO_3 at concentrations of 0, 0.01, 0.5, and 1.0 mol dm^{-3} for 60 min. and subsequently rinsed with water. Germination percentage was counted as described above.

Experiment 3

Seeds at each maturation stage were first immersed in KNO_3 (0 or 1 mol dm^{-3}) for 60 min., followed by rinsing with water before the germination assays, which were conducted in a roll of paper towel moistened with the following solutions: GA_3 at 0 and 50 mg dm^{-3} (1.4 x 10^{-4} mol dm^{-3}) and CEPA at 0 and 5 x 10^{-7} mol dm^{-3}. Seeds were then submitted to the following treatment-combinations:

T1:CEPA (0 mol dm^{-3}) + GA_3 (0 mg dm^{-3}) + $KN0_3$ (0 mol dm^{-3});

T2: CEPA (0 mol dm^{-3}) + GA_3 (0 mg dm^{-3}) + $KN0_3$ (1 mol dm^{-3});

T3: CEPA (0 mol dm^{-3}) + GA_3 (50 mg dm^{-3}) + $KN0_3$ (0 mol dm^{-3});

T4: CEPA (0 mol dm^{-3}) + GA_3 (50 mg dm^{-3}) + $KN0_3$ (1 mol dm^{-3});

T5: CEPA (5 x 10^{-7} mol dm^{-3}) + GA_3 (0 mg dm^{-3}) + $KN0_3$ (0 mol dm^{-3});
T6: CEPA (5 x 10^{-7} mol dm^{-3}) + GA_3 (0 mg dm^{-3}) + $KN0_3$ (1 mol dm^{-3});
T7: CEPA (5 x 10^{-7} mol dm^{-3}) + GA_3 (50 mg dm^{-3}) + $KN0_3$ (0 mol dm^{-3});
T8: CEPA (5 x 10^{-7} mol dm^{-3}) + GA_3 (50 mg dm^{-3}) + $KN0_3$ (1 mol dm^{-3}).

Four replicates of 50 seeds at each stage of maturation were used; the seeds were allowed to germinate as described in Experiment 1. Germination was monitored every two days during the 30 days of the experiment; seedlings were considered normal if they were at least 5 cm long. Germination percentage was counted at 14 and 30 days after sowing (BRASIL, 2009). The obtained data were used to estimate the Germination Index Speed (GIS apud MAGUIRE, 1962).

Biochemical assays

Seeds were taken from each of the treatments 48 hours after sowing, frozen in liquid nitrogen and stored at -80°C until required.

Four samples of 200 mg of fresh mass (approximately eight seeds) per treatment were used to estimate the soluble protein content. The samples were soaked in 2.0 cm^3 of the extraction medium (100 mmol dm^{-3} potassium phosphate buffer at pH 6.8, 0.1 mmol dm^{-3} EDTA, 0.1 mmol dm^{-3} DTT, and polyvinylpolypyrrolidone) 4% (w v^{-1}). The extract obtained was centrifuged at 15,000×g for 15 min. at 4°C, and the supernatant was collected for further analysis. The soluble protein concentration was determined according to Bradford (1976) using an ELISA reader (Tunable Microplate Reader, VERSAmax, Molecular Devices, Sunnyvale, USA).

Four samples (10 mg each) were used to estimate the organic and nitric nitrogen fractions. The seed samples were lyophilized (Liotop L101, São Carlos, Brazil) for 72 hours. The samples were subjected to sulfuric acid digestion, followed by treatment with the Nessler reagent for determination of organic nitrogen (JACKSON, 1958). Nitric nitrogen was determined according to the methods of Cataldo et al. (1975). The total nitrogen concentration was computed as the sum of these fractions (organic and nitric nitrogen). The percentage of total protein was estimated by multiplying the total nitrogen content by 6.25.

Four seed samples (25 mg each) were taken for quantifying lipids and carbohydrates. Crushed samples were added to Eppendorf tubes filled with 2 cm^3 of a mixture composed of chloroform (0.5 cm^3), methanol (0.5 cm^3) and water (1 cm^3). After centrifuging the extract for 5 min. at 4,000×g, the upper phase (chloroform) was used to quantify the lipids according to the method of Bligh and Dyer (1959), whereas the quantification of soluble sugars was performed using the lower phase (methanol + water). The remaining insoluble pellet was hydrolyzed with 3% HCl for 3 hours in a dry bath at 90°C for starch estimation. The soluble sugar content and the amount of starch hydrolyzed were determined colorimetrically using the anthrone reagent (YEMN; WILLIS, 1954).

Experimental design

For all experiments, the treatments were randomized with four replications and data were subjected to an analysis of variance in a factorial scheme. For experiments 1 and 2, the means of each treatment were compared using an *F*–test at 5% probability, and polynomial regressions were employed to examine the germination percentage as a function of the concentrations of CEPA or KNO_3. In experiment 3, factorial scheme 2 (maturation stages 3 and 5) x 8 (plant hormones treatment-combinations) was used; the means of each treatment were compared using the Scott-Knott test, and comparisons for each maturation stage were performed using the *F*-test. The threshold level for statistical significance was set at $\alpha = 0.05$. All statistical analyses were performed using SigmaPlot and SAS software.

Results and discussion

Experiment 1

On average, the final germination percentage of control seeds was approximately 57%. The germination percentage of seeds at stage 3 was unresponsive to CEPA concentrations ranging from 0 to 10^{-5} mol dm^{-3}; the germination percentage decreased with increasing CEPA concentrations (approximately 23% germination at the highest CEPA concentration), measured at both 14 and 30 days after sowing. In sharp contrast, no effect of CEPA on germination of the stage 5 seeds was observed (Figure 1).

Ethylene can promote the rupture of the endosperm and seems to maintain low concentrations of ABA (an inhibitor of seed germination; LINKIES et al., 2009), as found in seeds of *Arabidopsis thaliana* with a mutation in the ethylene receptor (*etr1-2*); these seeds display higher ABA concentrations compared to wild seeds (CHIWOCHA et al., 2005). However, conflicting results have been reported regarding the role of ethylene in seed dormancy. Whereas this hormone has been shown to play an important role in breaking dormancy in species such as sunflower

(BORGHETTI et al., 2002) and *Stylosanthes humilis* (RIBEIRO; BARROS, 2006), in passion fruit, ethylene alone did not increase germination (AMARO et al., 2009) and even reduced it when combined with GA (ZUCARELI et al., 2003). Here, we showed inhibition of germination of seeds treated with relatively high concentrations of CEPA (and presumably elevated concentrations of ethylene, also), which contrasts with the current view that regulators such as ethylene scarcely impair germination, as reviewed by Finch-Savage and Leubner-Metzger (2006). Because relatively high concentrations of CEPA were associated with decreased germination percentages, a lower concentration of CEPA (10^{-7} mol dm^{-3}) was used in Experiment 3, in combination with other growth regulators (see below).

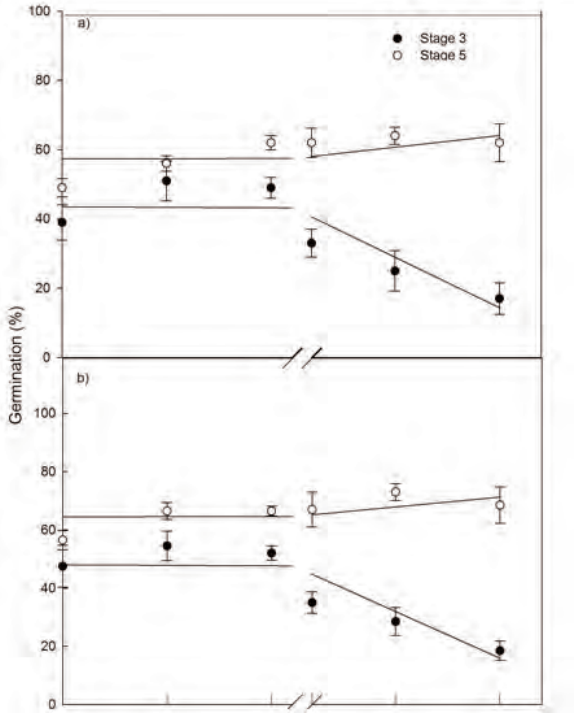

Figure1. Germination of seeds at stages 3 and 5, as affect by CEPA, monitored at (a)14 days after sowing – stage 3 ($y = -295.6\ x^{**} + 0.433$, $R^2 = 0.784$) and stage 5 ($y = 70.59\ x^{ns} + 0.569$, $R^2 = 0.246$); and (b) 30 days after sowing – stage 3 ($y = -320.8\ x^{**} + 0.479$, $R^2 = 0.826$) and stage 5 ($y = 67.47\ x^{ns} + 0.645$, $R^2 = 0.256$). $^{**}p \leq 0.01$, ^{ns}non significant ($p > 0.05$), $n = 4 \pm$ SE.

Experiment 2

KNO_3 is an important inducer of germination in papaya seeds (TOKUHISA et al., 2007a). KNO_3 at concentrations equal to or higher than 0.1 mol dm^{-3}, supplied for 60 min., overcame seed dormancy, more than doubling germination percentage 30 days after sowing (27% in controls and 61% for seeds treated with 0.1 mol dm^{-3} KNO_3) at both maturity stages (Figure 2). The effects of NO_3^- were similar to those mediated by NO and NO_2^- (BETHKE et al., 2006; NEILL et al., 2003). In fact, NO and KNO_3 act as signaling molecules in several plant species, and experiments have shown that these molecules are components of the signaling network that controls seed dormancy (FOOTITT et al., 2011; HANCOCK et al., 2011).

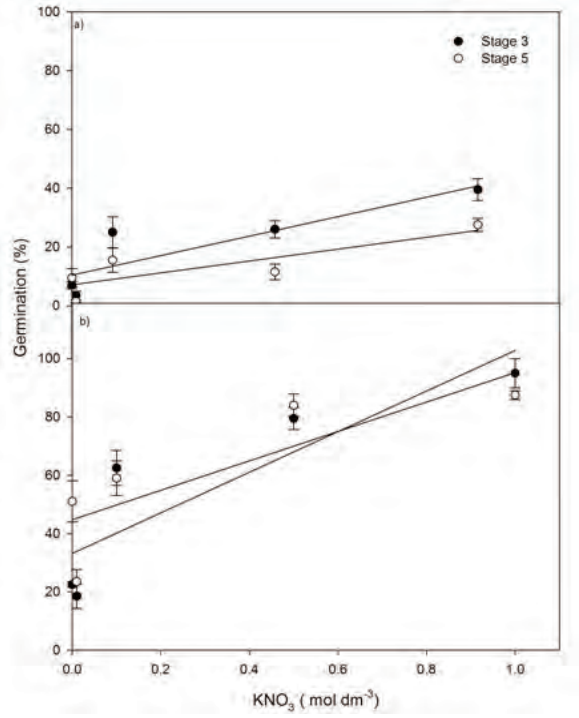

Figure 2. Germination of seeds at stages 3 and 5, as affect by KNO_3, monitored at (a) 14 days after sowing – stage 3 ($y = 0.303\ x^{**} + 0.104$, $R^2 = 0.774$) and stage 5 ($y = 0.185\ x^{**} + 0.071$, $R^2 = 0.699$); and (b) 30 days after sowing – stage 3 ($y = 0.696\ x^{**} + 0.331$, $R^2 = 0.775$) and stage 5 ($y = 0.587\ x^{**} + 0.383$, $R^2 = 0.749$). $^{**}p \leq 0.01$, and ^{ns}non significant ($p > 0.05$), $n = 4 \pm$ SE.

Experiment 3

Control seeds at both maturity stages showed similar germination percentages (approximately 10%) at the first counting. A higher percentage of germination was observed at 14 days after sowing for seeds at stage 3 in T2 (KNO_3 only) and T4 (treatment with GA_3 + KNO_3). However, the differences observed between seeds of the two stages at the beginning of the experiment (14 days), were no longer observed 30 days after sowing, except in T5 (CEPA only), when stage 3 seeds showed a higher germination percentage (Figure 3).

The combination of CEPA plus GA_3 (T7) was effective in overcoming dormancy of seeds at stage 5, resulting in germination percentages higher than 80%, similar to those obtained with

KNO_3. GA_3 alone (T3) was effective and almost doubled the germination of seeds at stage 5, from 32% in controls to 57% (T3). GAs are important growth regulators and have often been employed to break the dormancy of papaya seeds (LOPES et al., 2009; TOKUHISA et al., 2007b).

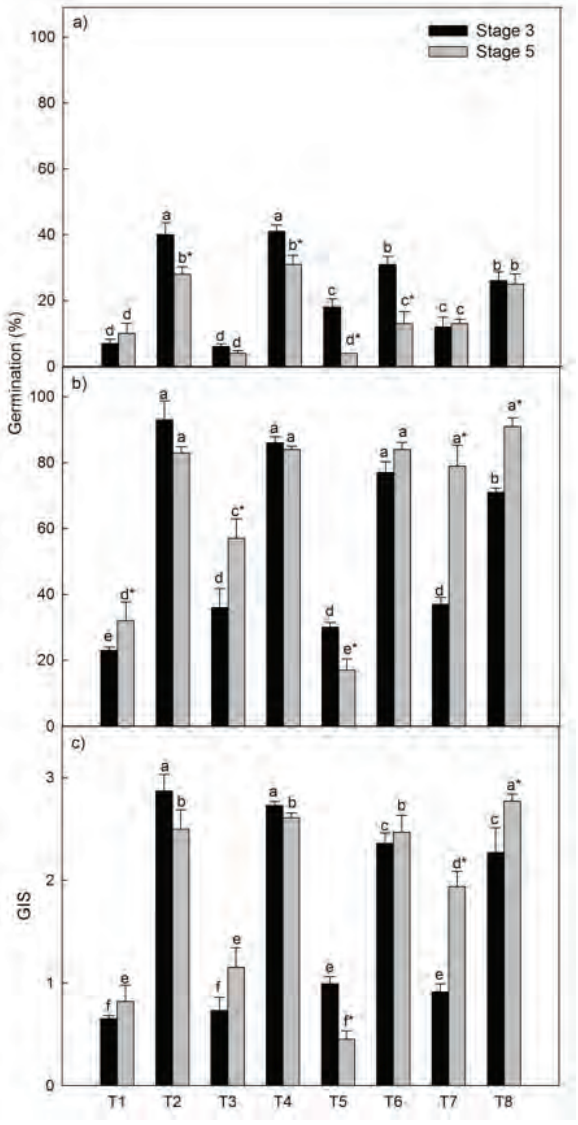

Figure 3. a) Germination rates at 14 days after sowing and b) 30 days after sowing, c) Germination Index Speed (GIS) obtained in seeds at maturity stages 3 and 5. T1: (CEPA (0 mol dm^{-3}) + GA_3 (0 mg dm^{-3}) + $KN0_3$ (0 mol dm^{-3}); T2: (CEPA (0 mol dm^{-3}) + GA_3 (0 mg dm^{-3}) + $KN0_3$ (1 mol dm^{-3}); T3: (CEPA (0 mol dm^{-3}) + GA_3 (50 mg dm^{-3}) + $KN0_3$ (0 mol dm^{-3}); T4: (CEPA (0 mol dm^{-3}) + GA_3 (50 mg dm^{-3}) + $KN0_3$ (1 mol dm^{-3}); T5: (CEPA ($5x10^{-7}$ mol dm^{-3}) + GA_3 (0 mg dm^{-3}) + $KN0_3$ (0mol dm^{-3}); T6: (CEPA ($5x10^{-7}$ mol dm^{-3}) + GA_3 (0mg dm^{-3}) + $KN0_3$ (1mol dm^{-3}); T7: (CEPA ($5x10^{-7}$ mol dm^{-3}) + GA_3 (50 mg dm^{-3}) + $KN0_3$ (0 mol dm^{-3}); T8: (CEPA ($5x10^{-7}$ mol dm^{-3}) + GA_3 (50 mg dm^{-3}) + $KN0_3$ (1 mol dm^{-3}). Means followed by same letter do not differ significantly from one another (Scott-Knott test, $p \leq 0.05$). *Denotes differences between seeds at stages 3 and 5 only within each treatment (a pair of rectangles) (F-test, $p \leq 0.05$). $n = 4 \pm$ SE.

The germination percentage of seeds at stage 5 (with GA_3 alone, T3) was 57%, whereas CEPA alone (T5) resulted in germination percentages as low as 17%. The combination of GA_3 and CEPA (T6) produced an additive effect, that is, germination percentage increased to 79% (Figure 3). According to Feurtado and Kermode (2007), in seeds of lettuce the thermodormancy promoted by storage at 32°C in the dark was not overcome by ethylene, GA or cytokinin applied singly. It is possible that GA_3 and ethylene act in concert to promote embryo growth and weakening of the tissues surrounding the radicle (FEURTADO; KERMODE, 2007). In *Arabidopsis* seeds, treatment with GA_4 stimulates germination and increases the expression of AtERS1 (ethylene response sensor), a component of the ethylene signaling pathway.

Seeds at both maturity stages showed lower concentrations of soluble sugars when treated with growth regulators, averaging 6.7% on a dry mass (DM) basis against approximately 12.5% DM in untreated seeds (Figure 4). This discrepancy might be related to the activation of pathways associated with reserve mobilization during the germination phase, when large amounts of energy are consumed for embryo growth and seedling establishment (BUCKERIDGE et al., 2000).

Seeds treated with KNO_3 alone (T2) exhibited the highest starch content and the lowest soluble sugar content at stage 3. The GIS and the first count of seed germination (14 days after sowing) were higher for seeds at stage 3 than at stage 5 (Figure 3). This indicates that KNO_3 can affect pathways associated with carbohydrate remobilization, which depended on the stage of maturation, as evidenced by an elevated consumption of soluble sugars in seeds at stage 3 (Figure 4).

The percentage of carbohydrates in untreated seeds (T1) was approximately 12.5% DM, a value close to the 11.7% DM reported by Marfo et al. (1986). The very low average starch content (0.35%) is consistent with anatomical studies showing that the endosperm of papaya seeds is apparently devoid of starch (SANTOS et al., 2009).

The highest concentrations of starch (0.5% DM) occurred in seeds treated with CEPA (T5 to T8), regardless of maturation stage (Figure 4). Ethylene appeared, therefore, to inhibit starch breakdown, leading to higher starch content in the seeds. According to Wuriyanghan et al. (2009), rice plants over-expressing the ethylene receptor *etr2* showed greater starch accumulation,

paralleling lower expression of the enzyme α-amylase.

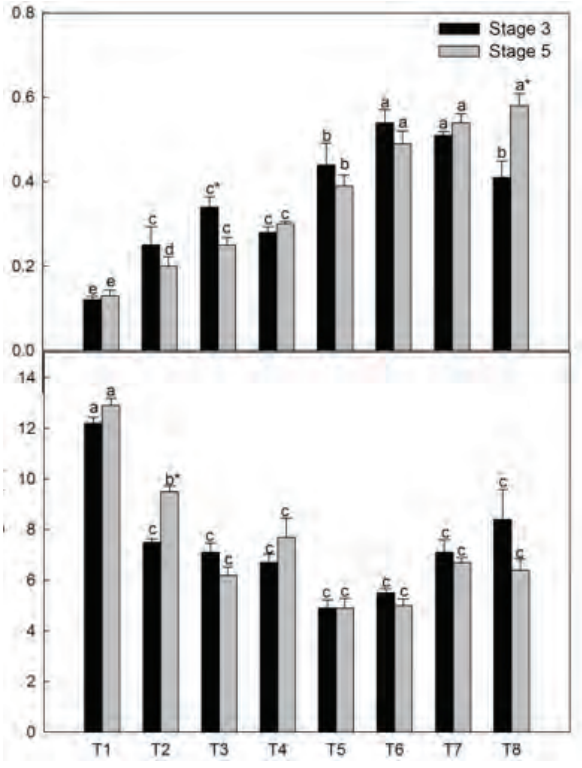

Figure 4. Starch and soluble sugar content (on a DM basis) in seeds at maturity stages 3 and 5, subjected to different treatments. See further details in the legend to Figure 3. Means followed by same letter do not differ significantly from one another (Scott-Knott test, $p \leq 0.05$). *Denotes differences between stages 3 and 5 only within each treatment (a pair of rectangles) (*F*-test, $p \leq 0.05$). $n = 4 \pm$ SE.

Lipids constitute important carbon stores that can form hexoses through gluconeogenesis. In addition, through β-oxidation, lipids participate in the formation of compounds, such as NADH and $FADH_2$, that can be used during seed germination (HELDT; PIECHULLA, 2011). The papaya seeds showed lipid levels close to 10% DM irrespective of stage of maturation, except in the T6 treatment (CEPA and KNO_3) (Figure 5). Regardless of maturation stage, lipid mobilization was chiefly apparent in seeds treated with GA_3 alone (T3) or with KNO_3 (T4) (Figure 5). According to Aya et al. (2009), the expression of genes related to lipid and secondary metabolism increased in GA-treated rice plants.

Concentrations of soluble and total proteins remained constant, regardless of maturity stage and treatment, and averaged 5.4% and 19.1% DM, respectively (Figure 6). Notably, the total protein concentrations in this study were lower than those obtained by Marfo et al. (1986) and Puangsri et al. (2005).

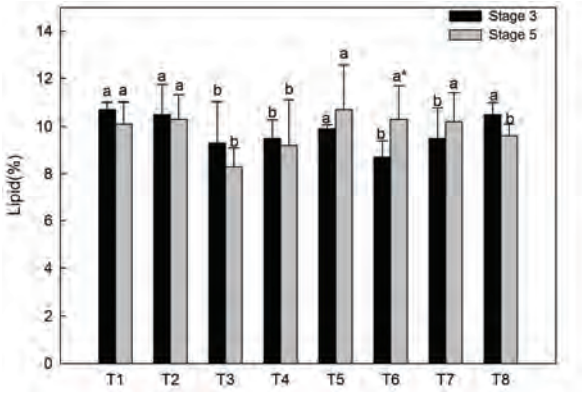

Figure 5. Lipid content (on a DM basis) in seeds at maturity stages 3 and 5 subjected to different treatments. See further details in legend to Figure 3. Means followed by same letter do not differ significantly from one another (Scott-Knott test, $p \leq 0.05$). *Denotes differences between stages 3 and 5 only within each treatment (a pair of rectangles) (*F*-test, $p \leq 0.05$). $n = 4 \pm$ SE.

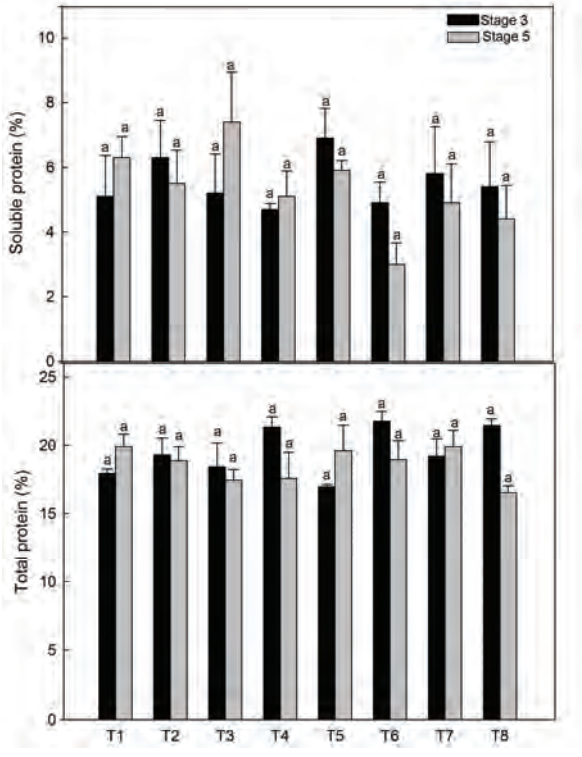

Figure 6. Soluble and total protein content (on a DM basis) in seeds of maturity stages 3 and 5, as affected by different treatments. See further details in legend to Figure 3. Means followed by same letter do not differ significantly from one another (Scott-Knott test, $p \leq 0.05$). *Denotes differences between stages 3 and 5 only within each treatment (a pair of rectangles) (*F*-test, $p \leq 0.05$). $n = 4 \pm$ SE.

During germination, seeds consume large amounts of protein and polysaccharide reserves (TONINI et al., 2010). In the present study, however, it seems that two days (after sowing) was not sufficient to characterize the consumption of protein reserves in papaya seeds. The seeds may have been consuming

energetic compounds, as suggested by the lower concentrations of soluble sugars (Figure 4), and had not yet begun biosynthesis of nitrogen compounds.

Conclusion

The control seeds of papaya taken from fruits at stage 5 displayed a high germination percentage 30 days after sowing, relative to stage 3 seeds. Treatment with CEPA impaired the germination of papaya seeds measured at 14 days after sowing, a fact apparently associated with decreased starch mobilization. Indeed, all other growth regulators tested were effective in overcoming seed dormancy. GA_3 alone or in combination with KNO_3 stimulated lipid mobilization. KNO_3 was the most effective compound in overcoming dormancy of papaya seeds.

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