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Seed treatment with aminobutyric acids improve carrot seed germination and vigor under thermal stress conditions

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Dissertation presented to obtain the degree of Master in Science. Area: Crop Science

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Dedication

To my family for their unconditional support and important influence in my education.

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SUMMARY

RESUMO

Tratamento de sementes com ácido aminobutírico melhora germinação e vigor de sementes de cenoura sob estresse térmico

Cenoura é uma cultura adaptada a diferentes condições de cultivo, tornando-se um dos vegetais mais cultivados do mundo. No Brasil, é cultivada no inverno e no verão, mas o clima extremo pode fazer com que os agricultores enfrentem problemas relacionados à germinação de sementes e ao desenvolvimento precoce das plantas, resultando na redução do plantio e da produtividade nos sistemas produtivos. No estágio vegetativo, o estresse térmico severo pode reduzir o crescimento, causar clorose e necrose foliar, senescência e até morte dos vegetais. A nível celular, o estresse térmico pode causar danos nas membranas, desnaturação proteica, inativação das enzimas mitocondrial e cloroplástica, síntese de proteínas e carboidratos prejudicada, degradação de proteínas, síntese de novas proteínas e inibição do metabolismo de carbono. Tornar as culturas tolerantes ao estresses ambientais é um desafio enfrentado pelos métodos de melhoramento clássico e transgenia, sendo rotineiramente experimentados e aplicados para aumentar a produtividade e a tolerância das culturas à estresses ambientais. Uma das maneiras mais simples promover tolerância a plantas sensíveis envolve o uso de biomoléculas com funções reguladoras ou estimulantes em plantas estressadas, os bioestimulantes. Bioestimulantes são definidos como substâncias que contêm compostos como microorganismos, inoculantes microbianos, ácidos húmicos, ácidos fúlvicos, hidrolisados de proteínas, aminoácidos e extratos de algas que estimulam o processo natural de crescimento das plantas. Alguns aminoácidos não proteicos, como ácido γ-aminobutírico (GABA), ácido β-aminobutírico (BABA), têm sido comumente estudados pela capacidade de conferir tolerância ao estresse ambientais. Com isso, o objetivo deste estudo foi avaliar a aplicação de um revestimento em sementes de cenoura composto com diferentes doses de GABA e BABA e avaliar seu efeito na germinação, emergência e vigor de sementes sob estresse térmico por calor e frio.

Palavras-chave: Sementes de cenoura, Tratamento de sementes, Altas temperaturas, Baixas temperaturas

ABSTRACT

Seed treatment with aminobutyric acids improve carrot seed germination and vigor under thermal stress conditions

Carrots are plants adapted to different growing conditions, becoming one of the most cultivated vegetables in the world. In Brazil, it has been cultivated both in winter and summer, but extreme weather can make farmers face problems related to seed germination and plant early development, resulting in the reduction of stand and yield in the productive systems. In the vegetative stage, severe thermal stress can result in retarded growth, chlorosis and leaf necrosis, senescence and even vegetable death. At the cellular level, heat stress can lead to membrane damage, protein denaturation, inactivation of mitochondrial and chloroplast enzymes, impaired protein and carbohydrate synthesis, protein degradation, new protein synthesis, and inhibited carbon metabolism. Making crops tolerants to environmental stress are challenges faced by classical breeding and transgenic approaches and different methods are routinely experimented and applied to enhance the productivity and stress tolerance of crops. One of the simplest ways of giving tolerance to sensitive plant species involves the use of biomolecules with regulatory or stimulating roles in stressed plants, the biostimulants. Biostimulants are defined as substances that contain compounds as microorganisms, microbial inoculants, humic acids, fulvic acids, protein hydrolysates, amino acids, and seaweed extracts that stimulate natural process of plant growth. Some non-protein amino acids, such as γ-aminobutyric acid (GABA), β-aminobutyric acid (BABA) have been commonly studied by the ability of confering tolerance to abiotic stress. With this, the objective of this study was to evaluate the application of a film coating in carrot seeds composed with different rates of GABA and BABA and evaluate it effect on seed germination, emergence and vigor under heat and cold stress.

Keywords: Carrot seeds, Seed treatment, Heat stress, Cold stress

1. INTRODUCTION

1.1. Carrot production and environmental stress

Carrot (*Daucus carota* L.) is a vegetable crop from the Apiaceae botanic family. It is a tuberous root crop largelly cultivated in the Brazil, mainly in the states of Minas Gerais, Goiás, Bahia, Rio Grande do Sul and Paraná (CEPEA, 2019). It is a high value crop and with this demand a considerable investiments on technology and agricultural inputs, also even though the main areas are suitable to use machinery, it still demand a high intense manpower generate with this considerable jobs in the production areas.

Carrots are adapted to different growing conditions, becoming one of the most cultivated vegetables in the world. In Brazil, it has been cultivated both in winter and summer, but extreme weather can make producers face problems related to seed germination and plant development, resulting in the reduction of stand and yield in the productive systems (PEREIRA et al., 2013). Many production systems are characterized by variable climate and soil, which can result in high risk conditions for agricultural producers (LIEBIG et al, 2007).

Agriculture is very vunerable to climate change. Changes in the average rainfall and temperature, interannual climate variability, shocks during specific phenological stages, and extreme weather events can ruin field and farmers (IPCC, 2012). There are crops that can tolerant more than the others some types of stresses, but in general environmental stresssignificantly affect crop production systems (SIMPSON, 2017). Environmental stress is the primary cause of global agricultural losses, reducing the production of most crops by more than 50% (PEÑA and HUGHES, 2007). Heat or cold temperatures inhibit plant performance at various organizational levels, resulting in altered responses at morphological, biochemical and molecular levels that reduce their growth potential (WAHID et al., 2007). For the most species show optimal growth within the range of 10-30 ºC, with significant reduction outside this range (WAHID et al., 2007).

In the vegetative stage, severe thermal stress can result in retarded growth, chlorosis and leaf necrosis, senescence and even vegetable death (NAYYAR et al., 2014). At the cellular level, heat stress can lead to membrane damage, protein denaturation, inactivation of mitochondrial and chloroplast enzymes, impaired protein and carbohydrate synthesis, protein degradation, new protein synthesis, and inhibited carbon metabolism (WAHID et al., 2007). As a defense mechanism, cells activate different enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), peroxidaseascorbate (APX) and glutathioneututase (GR) and non-enzymatic antioxidants such as ascorbic acid (ASA) and glutathione (GSH) (SAIRAM et al., 2000).

1.2. Biostimulants and seed treatment improving crop stress tolerance

Making crops tolerants to environmental stress are challenges faced by classical breeding and transgenic approaches and different methods are routinely experimented and applied to enhance the productivity and stress tolerance of crops. One of the simplest ways of giving tolerance to sensitive plant species involves the use of biomolecules with regulatory or stimulating roles in stressed plants (NAYYAR et al., 2014).

Biostimulants are defined as substances that contain compounds as microorganisms, microbial inoculants, humic acids, fulvic acids, protein hydrolysates, amino acids, and seaweed extracts that stimulate natural process of plant growth. (SHARMA et al., 2014). Among biostimulators, amino acids are considered vital in plant functioning, especially under stressful conditions, where they play a regulatory, antioxidant and signaling role, thus contributing to the best development in unfavorable temperature conditions, water supply, salinity stress and others (SHARMA and DIETZ, 2005).

Some non-protein amino acids, such as γ-aminobutyric acid (GABA), β-aminobutyric acid (BABA) have been commonly evaluated at the plant tolerance conference for abiotic stress. Jisha and Puthur (2016) found remarkable development of *Vigna radiata* seedlings subjected to osmotic and saline stress when their seeds were treated with BABA. GABA, in turn, has proven to be an effective method in conferring tolerance to tomato plants at low temperatures (MALEKZADEH et al., 2014).

Seed is the input that has significative sharing on carrot production, representing arounding 4-6% of its total cost (CEPEA, 2019). The use of tolerant hybrids seeds in the summer and winter time is necessary in order to prevent the effects of climate adiversities and get plants tolerant and productive under those conditions, resulting in yield and revenue to the farners. The seed treatment techonology involving the use aminoacids on its composition are mainly the priming technic and with this the exogenous application of these compounds in seed treatment has provided protection against various stresses in different plant species.

The use of aminoacids like BABA and GABA added to film coating technic has never been tested by researches, even though there are results comproving the effect of micronutrients, microoganisms or biostimulants added to the coating and its positive effect on seedling tolerance to thermal stress (BRADÁCOVÁ et al., 2016; GÓMEZ-MUÑOZ et al., 2018). Film coating seeds with amino acids is a viable alternative in the availability of these products in a way that provides greater technical efficiency in their use, and studies involving carrot seed treatment and its effect on seed germination and emergence is a relevant subject in order to improve plant standability and early growth development.

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2. SEED TREATMENT WITH AMINOBUTYRIC ACIDS IMPROVE CARROT SEED GERMINATION, EMERGENCE AND VIGOR UNDER HEAT STRESS CONDITION

Abstract

Carrot (*Daucus carota*) is a cool-season crop with plant stand establishment by direct seeding. This crop, when grown in tropical and subtropical climates, faces serious problems related to high temperatures in the seed germination, emergence, and early vegetative development. Biostimulants molecules have been currently used as an effective way of conferring stress tolerance in plants. Among the many products used, the nonprotein amino acids GABA (Gamma-aminobutyric acid) and BABA (Beta-aminobutyric acid) are selected priming agents for enhancing abiotic stress tolerance. However, the main studies with GABA and BABA application on improving plant stress tolerance are using them on spray application, nutrient solution and soil drench. Studies involving seed treatment associated with GABA and BABA are less common in the literature but could be efficient since it requires less input to treat a high volume of seeds. With this, the objective of this study was to evaluate the influence of film coating carrot seeds from the cultivar Erica F1 with different rates of BABA and GABA and its impact on seed germination and vigor under heat stress conditions (35 °C). Seed performace was determined by assessing seed Germination Percentage (GP), First count of Germination (FC), Germination speed index (GSI), Thermotolerance (THT), Dry weight of the seedlings (DW), Field emergence (FE), Greenhouse emergence (E), First count of greenhouse emergence (FCE), Emergence speed index (ESI) and Dry weight of seedlings from the greenhouse test (DWE) as well as the MDA and H_2O_2 content. The heat stress temperature drastically reduced seed germination and vigor parameters assessed under 35 °C. Treating the seeds with BABA and GABA at the rates of 0.50 and 2.00 mM, respectively, was possible to reduce the negative effect of heat stress on seed germination, emergence and vigor as well as the ROS activity.

Keywords: Carrot seeds, Seed treatment, Heat stress, BABA and GABA

2.1. Introduction

Carrot (*Daucus carota*) is a cool season crop with plant stand establishment by direct seeding. This crop when grown in tropical and subtropical climates faces serious problems related to high temperatures in the seed germination, emergence and early vegetative development. Many researchers have reported the occurrence of higher temperatures during the initial stages of development as the main cause of decreasing seed germination and emergence, uniformity and stand establishment, reflecting on yield loss and profitability (PEREIRA et al., 2007; NASCIMENTO et al., 2008; NASCIMENTO et al., 2013).

Reactive oxygen species (ROS) are normally produced by plants, however under stress conditions those compounds are significantly increased above the plant capacity to scaving. Heat stress generate such as lipid peroxides, singlet oxygen, superoxide radicals, hydroxyl radicals, and hydrogen peroxide (H_2O_2) that are able to damage pigments, nucleic acids, proteins, and membranes, aggravating the cellular injury and leading on cell death (LIU and HUANG, 2000; NAYYAR et al., 2014; XU et al., 2006).

Due to the relevance of growing this crop on tropical and subtropical climates and the challenges faced by classical breeding and transgenic approaches to produce tolerant plants, different methods are routinely experimented and applied to enhance the productivity and stress tolerance of crops, and one of the most recurrent forms is the use of biomolecules with regulatory or stimulating roles (NAYYAR et al., 2014).

Biostimulants are defined as substances that contain compounds as microorganisms, microbial inoculants, humic acids, fulvic acids, protein hydrolysates, amino acids, and seaweed extracts that stimulate natural process of plant growth. (SHARMA et al., 2014). Among biostimulators, amino acids are considered vital in plant functioning, especially under stressful conditions, where they play a regulatory, antioxidant and signaling role, thus contributing to the best development in unfavorable temperature conditions, water supply, salinity stress and others (SHARMA and DIETZ, 2005).

Among the many products used, the nonprotein amino acids GABA (Gamma-aminobutyric acid) and BABA (Beta-aminobutyric acid) are selected priming agents for enhancing abiotic stress tolerance (NAYYAR et al., 2014; VIJAYAKUMARI et al., 2016). GABA is a four carbon non-protein amino found in all prokaryotic and eukaryotic organisms and its functions involved the cytosolic pH regulation, carbon metabolism, nitrogen storage, transport, plant development, and plant defense (ROBERTS, 2007; BARBOSA et al., 2010; VIJAYAKUMARI et al., 2016).

According to Kinnersley and Turano (2000), GABA can act as a signal stress promoter, and with this can induce answer to plants produce self-defense against the faced condition. It also can work as an osmolyte or even support the synthesis of some of them (SHELP et al., 1999). Many researchers have been studying the effect of GABA on plant stress tolerance in different species and ways of application with consistent results on plant tolerance. Malekzadeh et al. (2014), when applied GABA (750 μM/L) by spray application on tomato seedlings (*Lycopersicon esculentum*) had beneficial effects on reducing chilling stress by increasing growth of seedlings and antioxidant enzyme activity, as well as osmolyte such as proline and soluble sugar content. Nayyar et al. (2014) had similar results when studied the effect of GABA application on nutrient solution (1 mM) in rice (*Oryza sativa*) under heat stress condition, improving growth and survival rate, reducing membrane injury and damage to mitochondrial and chloroplast function, and improving the antioxidant capability of plants to scavenge ROS species.

Another amino acid that has been showing interesting results on plant stress tolerance is BABA. It is an isomer of GABA that is synthetic produced and very rare found on naturally

(JAKAB et al., 2005; MAYER et al., 2006). Reports that investigated the effect of BABA application on increasing stress tolerance under drought and salinity (JAKAB et al., 2005; MACARISIN et al., 2009), osmotic stress (JISHA AND PUTHUR, 2016) and heat (ZIMMERLI et al. 2007; WU et al., 2010). Zimmerli et al. (2007), when studied the ability of Arabidopsis seedlings on overcoming the heat tolerance found that plants primed with BABA (0.5 mM) applied on nutrient solution had increased the survival rate as compared with the control treatment without BABA.

However, the main studies with GABA and BABA application on improving plant stress tolerance are using them on spray application, nutrient solution and soil drench. Studies involving seed treatment associated with GABA and BABA are less common in the literature. The film coating technic is a good way of apply plant protection products, biologicals and stimulants all within a thin film coat layer, reducing cost and products need, and positively impacting the environment. With this, the objective is to assess the effect of film coating with aminobutyric acids on carrot seed germination and vigor under high temperature stress.

2.2. Material and methods

2.2.1. Seed treatment

The seeds of carrot (*Daucus carot*) cv. Erica F1 were provided by Agristar do Brasil Inc., and was used for all studies. The film coating composition was made by applying per kg of seeds 20 $ml.Kg^{-1}$ of Maxim XL® (fungicide), 30 $ml.Kg^{-1}$ of Filmcat L083® (Incotec polymer), 30 $ml.Kg^{-1}$ of water and addition or not of amino acid in the following rates. The rates of BABA and GABA (Sigma Aldrich): 0.25, 0.50, 1.00 and 2.00 mmol L^{-1} were added to the film coating composition, seeds with polymer but no amino acid applied were used as the control. The formulations were applied using a laboratory scale conventional pan coater allowing a better uniform treatment application on coverage carrots seeds.

2.2.2. Effect of temperature on carrot seed germination

In order to assess temperatures, the cause heat stress, seeds of carrot were submitted to germinate under different temperatures: 20, 25, 30, 35, and 40ºC in BOD (Biochemical Oxygen Demand) chambers with light (12 h)/dark (12 h) photoperiod. Seeds were placed in light transparent plastic boxes (11 x 11 x 3.5 cm.) containing two sheets of blotting paper moistened with 14 ml of water. Each treatment was replicated four times and 50 seeds for each replicate. The germination rates were counted after 14 days according to the recommendations from the rules for seed testing (BRASIL, 2009) with results expressed as percentage of seed germinated.

2.2.3. Effect of BABA and GABA on seed germination and vigor under heat stress

Germination test. The treated and non-treated seeds with BABA and GABA were placed in light transparent plastic boxes (11 x 11 x 3.5 cm.) containing two sheets of blotting paper moistened with 14 ml of water and kept at 20 (ideal temperature) or 35°C (stress temperature) at 12 h light/dark photoperiod. The 35°C temperature was chosen based on the preliminary tested described at 2.2 item. Each treatment was replicated four times with 50 seeds for each replicate. The germination at the 7d (*First count of germination*), and 14 days (*Total germination*) were obtained according to the recommendations from the rules for seed testing (BRASIL, 2009).

Emergence of seedlings in greenhouse: The treated and non-treated seeds with BABA and GABA were sowed in four replications of 50 seeds in a multicellular expanded polystyrene tray containing the commercial substrate made from pine bark and placed in a greenhouse with 12 h light/dark photoperiod. Evaluations occurred after seven and 14 days of sowing, and the results were expressed as an average percentage of emerged seeds.

Field emergence of seedlings: Four replicates of 50 seeds for each treatment were sown in furrows 5.0 m long spaced 0.20 m apart and 0.01 m deep. Soil moisture was kept sufficiently wet by drip germination. The percentage of emerged seedlings was counted on the 21th day after sowing.

Germination and emergence speed index. The germination speed of the carrot seeds was determined based on the increase of the number of new seedlings counted daily throughout the test period. The following the formula was used (MAGUIRE, 1962):

GSI = (N1 / D1) + ... + (Nn / Dn); Where Nn = number of normal seedlings counted daily; Dn = days after test installation.

Dry weight of seedlings. At the end of the 14 days (germination and emergence test) and 21 days (field emergence test) samples of each replication were placed in an oven run at 80 ºC for 48 h in order to obtain the dry weight of seedling. The results were expressed in mg. seedling^{-1}.

2.2.4. Effect of BABA and GABA on antioxidant capacity of seeds under heat stress

Lipid peroxidation and H_2O_2 content. Treated seeds with BABA (0.50 g.Kg^{-1} of seeds) and GABA (2.00 g.Kg^{-1} of seeds), as well as the control treatment (0.00 g.Kg^{-1} of seeds) were collected at 6 days after sowing, frozen in liquid nitrogen and stored at -80 ºC for biochemical analysis. The rates were selected based on previous germination and vigor testes.

Lipid peroxidation was determined by measuring the content of Malondialdehyde (MDA), which was extracted with 0.25% thiobarbituric acid (TBA) in the absorbance at 532 and 600 nm and results expressed at mmol.g^{-1} of fresh matter (HEATH and PACKER, 1968). The H_2O_2 content was determined according to methodology described by Alexieva et al., (2001) with hydrogen peroxide (H_2O_2) standard curve. Seed material samples (0.1 g) were macerated in 0.1% (m/v) of trichloroacetic acid and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant (1 ml) was mixed with 1 mL of 100 mM potassium phosphate buffer (pH 7.5). The absorbance was read at 390 nm and the results expressed at mmol. g^{-1} of fresh matter.

2.2.5. Statistical analysis

The statistical analysis of the results obtained in the evaluations were submitted to variance analyses by the F test. The treatment means were compared by the Tukey test ($p < 0.05$) using the R package stats (version 3.5.3) (R Core Team, 2019).

2.3. Results

2.3.1. Effect of temperature on carrot seed germination

The germination percentage as a function of different temperatures is presented in figure 1. The germination percentage decreased as a function of increasing temperatures. Temperatures that ranged between 20 and 30 ºC did not affect seed germination of cultivar Erica F1, showing percentage of germination values of 84, 81, and 82% for the temperatures of 20, 25, and 30 °C respectively. Differently, temperatures above 30 ºC drastically reduced seed germination. The temperatures of 35 and 40°C were responsible for 60% and 100% reduction as compared to the 20 °C temperature. With this, the temperatures of 20 and 35 ºC were considered as optimal and heat stress temperatures for carrots seed germination. The 20 °C is also recommended by the rules for seed testing and analysis as an optimal test temperature (BRASIL, 2012).

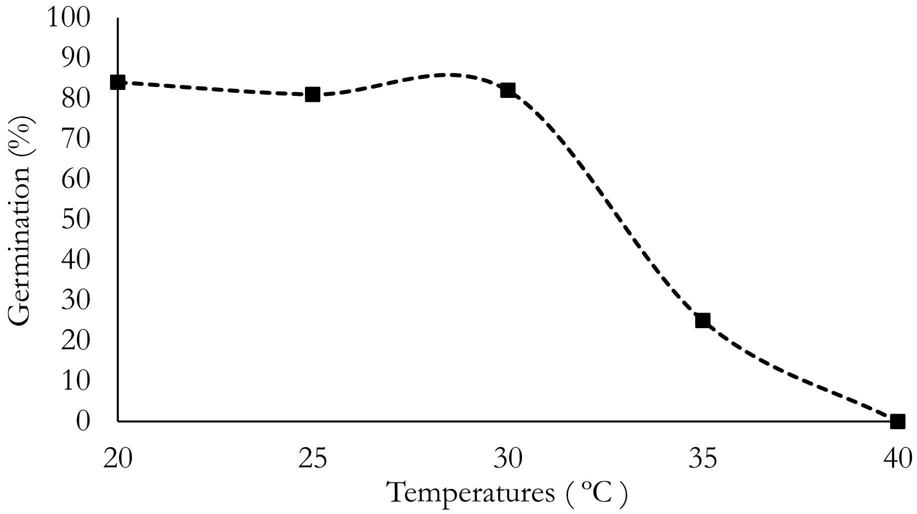

Fig. 1. Effect of different temperatures on carrot seed germination (Erica F1)

2.3.2. Effect of BABA and GABA application on seed germination and emergence

The Germination percentage at 20 °C (GP20), Germination percentage at 35 °C (GP35), First-count germination at 20 °C (FG20), First-count germination at 35 °C (FG35), Germination speed index at 20 °C (GS20), Thermotolerance (THT), Germination speed index at 35 °C (GS35), Dry weight of seedlings at 20 °C (DW20), Dry weight of seedlings at 35 °C (DW35) for non-treated and treated seeds with BABA and GABA are presented on the table 1.

Table 1. Germination percentage at 20 °C (GP20) and 35 °C (GP35), First-count germination at 20 °C (FG20) and 35 °C (FG35), Germination speed index at 20 °C (GS20) and 35 °C (GS35), Thermotolerance (THT), Dry weight of seedlings at 20 °C (DW20), and 35 °C (DW35) of carrot seeds treated and non-treated with BABA and GABA.

TRAT	GP20	GP35	FC20	FC35	THT	GIS20	GIS35	DW20	DW35
	%		%		T35/T20	Index		mg	
CK1	75 b	28 c	62 ab	53 ns	0.37 c	5.58 c	4.01 ns	0.69 ns	0.24 c
CK2	85 a	28 c	62 ab	50 ns	0.33 c	6.24 a	3.94 ns	0.77 ns	0.32 bc
B - 0.25	83 ab	52 b	54 b	53 ns	0.63 ab	5.77 bc	3.85 ns	0.81 ns	0.38 ab
B - 0.50	90 a	66 a	66 a	50 ns	0.73 a	6.54 a	4.19 ns	0.72 ns	0.42 a
B - 1.00	84 a	52 b	67 a	54 ns	0.62 ab	6.31 a	3.57 ns	0.70 ns	0.47 a
B - 2.00	85 a	59 a	58 ab	54 ns	0.69 a	6.10 ab	3.93 ns	0.80 ns	0.38 ab
CV%	4.16	9.72	6.78	8.24	6.65	3.66	7.70	8.83	12.24
CK1	75 b	28 c	62 ns	53 ab	0.37 c	5.58 b	4.01 cd	0.69 ns	0.24 b
CK2	85 a	28 c	62 ns	50 b	0.33 c	6.24 ab	3.94 cd	0.77 ns	0.32 ab
G - 0.25	84 ab	57 a	65 ns	40 c	0.68 a	6.38 a	3.50 d	0.78 ns	0.36 a
G - 0.50	82 ab	42 b	64 ns	61 a	0.51 ab	6.06 ab	5.46 a	0.68 ns	0.34 ab
G -1.00	88 a	50 ab	67 ns	55 ab	0.57 ab	6.54 a	4.42 bc	0.75 ns	0.32 ab
G - 2.00	87 a	58 a	65 ns	58 ab	0.67 a	6.34 a	4.90 ab	0.74 ns	0.31 ab
C.V%	3.86	4.06	6.50	7.64	5.54	4.47	7.06	7.12	13.34

Means with the same letter within a column were not different by Tukey test ($p < 0.05$).

Germination percentage (GP): Under optimal condition (20 °C), the percentage of germination of carrots seeds treated with BABA and the film-coated control (CK2) were responsible for the best results, and were not statistically different among each other. The control treatment (CK1) had the lowest results for seed germination under the above-mentioned condition but did not differ from the rate of 0.25 mM. When the seeds were submitted to heat stress temperature, the BABA treatments were superior to the controls (CK1 and CK2), and the best results were found for seeds treated with 0.50 and 2.00 mM. Those rates increased the germination percentage in 38 and 31% as compared to the CK1 control. Under optimal condition, seeds treated with GABA at the rates of 1.00, 2.00 and the CK2 control were responsible for the best results, while the control CK1 had the lowest values for carrot seed germination. The rates of 0.25 and 0.50 mM did not differ either from the CK1 control or the best results. Under heat stress condition the rates of 0.25 and 2.00 mM were responsible for the best results, increasing the germination percentage in 29 and 30% as compared to the CK1 and CK2 controls, which had the lowest values of germination percentage.

First count of germination (FC) of seeds treated with BABA under 20 °C at the rates of 0.50 and 1.00 mM were responsible for the best results, but did not differ statistically from the controls CK1, CK2, and the 2.00 mM. The rate of 0.25 mM showed the lowest result for this parameter. Under thermal stress, there was no statistical difference among the treatments with BABA. In the same way, seeds treated with GABA showed no statistical difference under optimal conditions, but when they were submitted to 35 °C the rate of 0.50 mM was responsible for the best results although it did not differentiate from the rates of 1.00, 2.00 mM and the control CK1. The rate of 0.25 mM showed the lowest results for this parameter.

The *Thermotolerance* (THT) was significantly increased by the treatments with BABA and GABA as compared to the controls. The BABA application at a rate of 0.50 and 2.00 mM increased the thermotolerance of seeds to germinate under thermal stress in an average of 102% as compared to the controls CK1 and CK2 but were not different from the 0.25 and 1.00 mM. In the same way, GABA seed treatments showed a similar behavior on the thermotolerance variable and its application increased the tolerance of seeds to germinate under heat stress. For all the rates assessed, the GABA application treatments were superior to the controls CK1 and CK2. The best results were obtained when the seeds were treated with 0.25 and 2.00 mM, even though they did not differ statistically from the rates of 0.50 and 1.00 mM.

The highest *Germination speed index* (GSI) under the optimal condition for BABA treatments was reached with the rates of 0.50, 1.00 mM and the CK2 control, even though they did not differ from the treatment of 2.00 mM. The lowest results under this condition were expressed by the control treatment CK1 and the rate of 0.25 mM. Under heat stress, the BABA treatment did not show a statistical difference between the controls. Seeds treated with GABA at the rates of 0.25, 1.00 and 2.00 mM were responsible for the best results, while the CK1 control the lowest values for this parameter. The rate of 0.50 and the CK2 control did not differ either from the best or the lowest values for the GSI under optimal condition. Under thermal stress, however, the GABA treatments at the rates of 0.50 and 2.00 mM showed superior results as compared to the other treatments, and the rate of 0.25mM, controls CK1 and CK2 had the lowest results for the GSI35.

The *Dry weight of seedlings* (DW) under optimal condition from seeds treated with BABA and GABA did not differ from the controls CK1 and CK2. Under thermal stress, however, the BABA treatments at the rates of 0.50 and 1.00 mM had the highest DW35 but did not differ from the 0.25 and 2.00 mM treatments. The controls CK1 and CK2 had the lowest DW35. The GABA treatments at the rate of 0.25 had the best results for this parameter but did not differentiate from the other GABA rates and the CK2 control. The CK1 control had the lowest results for this variable but did not differ from the rates of 0.50, 1.00, 2.00 mM and CK2 control.

The results from carrot seed field emergence (FE), greenhouse emergence (E), greenhouse first count of emergence (FCE), emergence speed index (ESI), and dry weight of seedlings of the emergence test are presented in table 2. *The field emergence* was positively affected by the film coating treatments with BABA and GABA compared to the control treatments CK1 and CK2. When the seeds were film coated with BABA at the rates of 0.50 and 1.00 mM, they increased the seedling emergence in 19 and 21% for the respective treatments compared to the control CK1, however, they did not differ statistically from the rates of 0.25 and 2.00 mM. The seeds treated with GABA showed the best results regardless of the rate used and compared to the controls for the field emergence parameter. By treating the seeds with GABA, it was observed that the field emergence was increased on average of 15% compared to the CK1 treatment.

Another parameter that was positively improved by the film coating treatment with BABA and GABA was the greenhouse emergence. It was observed that the treatments with BABA regardless of the rate analyzed had a positive effect on emergence of carrot seedlings in the greenhouse conditions, increasing on average of 8.5% compared to the control CK1. For the GABA treatment, the rates of 1.00 and 2.00 mM were responsible for the best results for greenhouse emergence as compared to the controls Ck1 and CK2, but did not differ statistically from the rates of 0.25 and 0.50 mM.

The First count of greenhouse emergence by the seeds that received BABA and GABA on its film coating composition was increased by the treatment of BABA and GABA. The rates of 0.25, 0.50 and 1.00 mM for the BABA treatment were responsible for the best results as compared to the control CK1 but did not differ statistically from the CK2 and 2.00 mM treatments, increasing on average 17% as compared to the control treatment CK1. The treatments that had GABA on its composition showed no statistical difference between the controls CK1 and CK2, thus GABA treatment showed no effect on the first count of greenhouse emergence. In the same way, the *greenhouse emergence speed index* did not show statistical difference among the treatment regardless of BABA, GABA and the controls CK1 and CK2.

The *Dry weight of seedlings* from the greenhouse emergence test was significantly increased by the treatments that had BABA and GABA on its composition. For the BABA treatments, the rate of 1.00 mM was responsible for the best results and differed from all the rates tested and the controls CK1 and CK2, increasing the dry weight of seedlings in 17.53 mg as compared to the control CK2 control that had the lowest values. GABA treatments also had a greater dry weight of seedlings. The rate of 2.00 mM showed the best results for this parameter (46.69 mg), but did not differ from the other GABA rates and so the control with film coating (CK2). However, when it is compared to the control treatment CK1, the GABA treatment of 2.00 mM increased in 13.36 mg the dry weight of seedlings.

Table 2. Field emergence (FE), Greenhouse emergence (E), First count of greenhouse emergence (FCE), Emergence speed index (GSIE) and Dry weight of seedlings of carrot seeds treated and non-treated with BABA and GABA

TRAT	FE	E	FCE	ESI	DWE
		%		Index	mg
CK1	36 c	66 b	46 b	4.00 ns	33.33 bc
CK2	43 c	65 b	56 ab	4.09 ns	31.67 c
B - 0.25	47 ab	74 a	66 a	4.69 ns	32.40 bc
B - 0.50	55 a	72 a	66 a	4.63 ns	37.60 bc
B - 1.00	57 a	76 a	65 a	4.53 ns	49.20 a
B - 2.00	50 ab	76 a	57 ab	4.52 ns	39.55 b
CV%	8.15	5.95	10.86	7.60	7.61
CK1	36 b	66 bc	46 ns	4.00 ns	33.33 b
CK2	43 b	65 c	56 ns	4.09 ns	31.67 ab
G - 0.25	52 a	72 ab	58 ns	4.39 ns	41.95 ab
G - 0.50	49 a	71 ab	52 ns	4.26 ns	44.35 ab
G -1.00	52 a	74 a	52 ns	4.41 ns	40.01 ab
G - 2.00	50 a	77 a	56 ns	4.56 ns	46.69 a
C.V%	7.84	4.14	10.42	9.18	15.39

Means with the same letter within a column were not different by Tukey test ($p < 0.05$).

2.3.3. Effect of BABA and GABA application on antioxidant capacity of seeds under heat stress

The Hydrogen peroxide content in carrot seeds presented in figure 3 did not have a statistical difference between the treatments, under stress and optimal condition. For the MDA content, it was observed that under optimal conditions there was no difference among the treatments. However, when the seeds were submitted to stress temperature, the MDA content was reduced in an average of 2.7 times with BABA and GABA application at the rate of 0.50 and 2.00 mM, respectively.

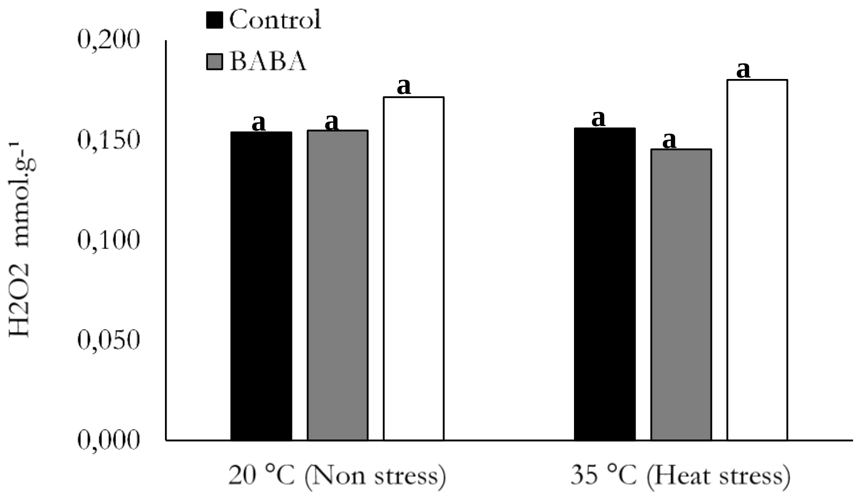

Fig. 2. Hydrogen peroxide (H2O2) in carrot seeds after treatment with GABA, BABA and control under optimal condition (20 °C) and heat stress (35 °C).

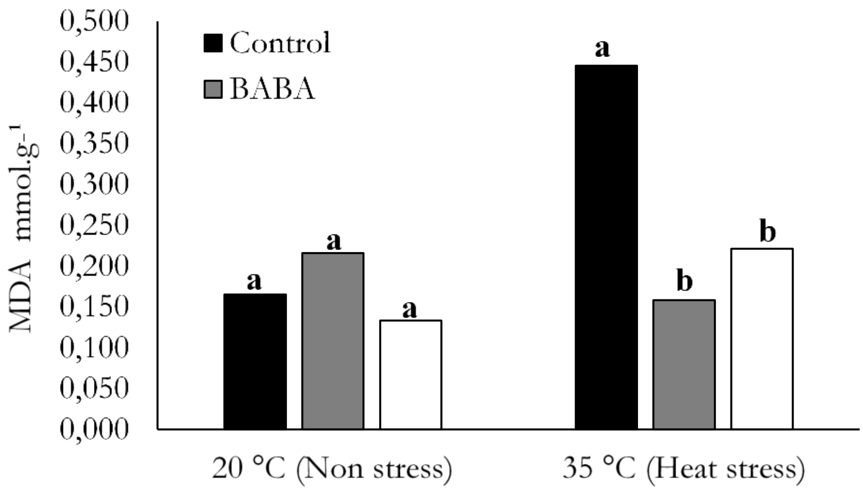

Fig. 3. Lipid Peroxidation (MDA) in carrot seeds after treatment with GABA, BABA and control under optimal condition (20 °C) and heat stress (35 °C).

2.4. Discussion

Temperature is an important factor in seed germination because it can inhibit the radicle emergence and affect seedling growth under thermal stress (PROBERT, 2000; ASHRAF AND

FOOLAD, 2005). According to Nascimento et al., (2013A, 2013B), heat stress can cause an excessive increase in seed respiration and metabolic activities of the seeds. Therefore, an optimal temperature should be provided during the germination process in order to make their cells metabolically active organized to seed germination and emergence (NASCIMENTO and PEREIRA, 2007). For carrot seeds, the temperature optimal range is between 20 and 30 ºC and above of this condition, the seed germination declines gradually. Our results show that heat stress significantly reduced the percentage of germination, first count of germination, germination speed index, and dry weight of seedlings. Treating seeds with BABA and GABA made them perform better and alleviated the heat stress effects. The BABA treatment positively affects seed germination percentage, thermotolerance and the dry weight of the seedlings under heat stress (35 °C). GABA treatments had a positive effect on all the parameters assessed in the germination test.

Many researchers have found on exogenous application of BABA and GABA an efficient and inexpensive method on promoting plant stress tolerance under environmental stresses like salt, hydric, cold and heat, once it requires small rates to promote the best effects. Li et al. (2016), studying the foliar application of GABA on promoting heat and drought tolerance to creeping bentgrass (*Agrostis stolonifera*) found that it significantly improved cell membrane stability, delayed leaf senescence, and enhanced the osmotic adjustment. Nayyar et al. (2014), studying heat stress on rice seedlings found that exogenous GABA applied at the rate of 1 mM was responsible for improving growth and survival of the seedlings.

The application of amino butyric acids like BABA and GABA on seed treatment aiming to promote stress tolerance is not very diffused, and there are no studies involving the use of film coating as a method of applying those products. With this, it is necessary to study the suitable rate to treat the seeds in order to obtain the best results provided by the amino acids.

According to the results obtained by Jisha and Puthur (2016), it was found that higher concentrations of BABA can cause a reduction of seedling growth and development, so low concentrations were more suitable for seed priming. Our results with the rates assessed could not define a rate of BABA as accurately as Jish and Puthur (2016) since the highest rate of 2.00 mM also showed great results, but the rate of 0.50 had a positive effect on the majority of the parameters. For treatments involving the use of GABA, Cheng et al. (2018) studied the use of seed priming treatment in white clover seeds under salt stress conditions and found that rates varying from 0.5 to 2.5 mM were responsible for the best results on salt stress tolerance, whereas high concentration of GABA (5 mM) inhibited seed germination. Those results are according to the obtained in this study, where rates of 0.50 and 2.00 mM were responsible for the best results on germination and vigor of carrot seeds.

The thermotolerance parameter, obtained by the ratio between the germination percentages obtained at high (35°C) and optimal temperature condition (20°C) shows the ability of cultivars to germinate under higher temperatures. The more is the percentage of germination under heat stress the greater is the thermotolerance of the cultivar. The control treatments had a thermotolerance ratio in average of 0.35 in this study, and even though the variety used can be considered a tolerant hybrid to high temperatures, the thermotolerance ratio is very low when it is compared to results obtained by Nascimento et al. (2008) when analyzed more than 60 genotypes, and obtained values above 0.60 for the open-pollinated varieties Brasilia, Esplanada and Alvorada. The use of BABA and GABA as a component of film coating allowed seeds to get more tolerant to higher temperatures and increased thermotolerance values to above 0.60.

High temperatures cause excessive accumulation of ROS and lipid peroxidation in plants and the success of the seed germination process depends on antioxidant protection against ROS especially during the early stages of development (BAILLY, 2004. CHEN et al., 2010). BABA and GABA are important ROS scavenging in plants and it has been scientifically proved those amino acids when exogenously applied could significantly improve multiple antioxidant enzyme activities reducing with this the oxidative damage. Under stress conditions, the generation of ROS leads to membrane damage and lipid peroxidation, resulting in an excessive increase in the MDA content (MITTLER et al. 2004; CARILLO, 2018).

High amounts of MDA in carrot seedlings heat-stressed (control) reveals that lipid peroxidation increase as cause of increased ROS generation, and it was observed that by treating seeds with BABA and GABA the MDA content was significantly reduced as compared with the control. This reduction in MDA content was reported by many researches, Jisha and Puthur (2016) when studied seed priming with BABA in *Vignia radiata* seeds found out a reduction in MDA under NaCl-stressed (32 %) condition and under PEG-stressed (58 %) condition when compared to the seedlings raised from non-primed seeds. In the same way, Malekzadeh et al. (2014) related the reduction on MDA content to alleviate the damage of tomato seedlings under chilling stress treated with GABA.

Under field conditions, the variables that can affect seed germination, emergence and vigor are uncountable and difficult to isolate. As compared to the controlled conditions studied, we could observe that field and greenhouse conditions drastically reduced carrot seeds emergence. Carrot crop is established by direct sowing and a low stand can occur when sowing is performed during unfavorable temperatures. Much research reports the negative effects of high temperatures on the establishment of the carrot stand. High temperatures may decrease or inhibit seed germination in the field and reduce stand uniformity (NASCIMENTO et al., 2013A, 2013B). Treating seeds with

BABA and GABA through film coating is an inexpensive strategy that can bring gains on plant stand, and consequently yield increasing, reducing costs and increasing return on investments.

2.5. Conclusion

In conclusion, we could find in this study that temperatures above 35 °C drastically impact germination and vigor of carrot seeds. Treating seeds with BABA and GABA at the rates of 0.50 and 2.00 mM respectively, as a component of the film coating treatment can improve carrot seed germination and vigor under heat stress and field conditions. Treating seeds with BABA and GABA under heat stress conditions contribute to reducing ROS activity assessed by the MDA content.

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3. SEED TREATMENT WITH AMINOBUTYRIC ACIDS IMPROVE CARROT SEED GERMINATION, EMERGENCE AND VIGOR UNDER COLD STRESS CONDITION

Abstract

Cold stress is one of the most important and limiting environmental stress, and it can cause tremendously damage on physiological and biochemical systems of the plants. The germination process in order to proceed well needs a favorable condition of temperature, which can allow the metabolic reactions to occur, and for the most cultivated species the optimal temperature ranges from 20 – 30°C, been temperatures below 15°C considered extreme for many vegetables' species. Biostimulants have been currently used as an alternative to confer stress tolerance in plants. The nonprotein amino acids like BABA (Beta-aminobutyric acid) and GABA (Gamma-aminobutyric acid) have been studied in this proporse. Studies involving seed treatment associated with BABA and GABA are less common in the literature. With this, the objective of this study was to evaluate the influence of film coating of carrot seeds from the cultivar Amanda F1 with different rates of BABA and GABA and its impact on seed germination and vigor under cold stress conditions (10 °C). Seed performace was determined by assessing seed Germination Percentage (GP), First count of Germination (FC), Germination speed index (GSI), Thermotolerance (THT), Dry weight of the seedlings (DW), Field emergence (FE), Greenhouse emergence (E), First count of greenhouse emergence (FCE), Emergence speed index (ESI) and Dry weight of seedlings from the greenhouse test (DWE) as well as the MDA and H_2O_2 content. The cold stress temperature significantly reduced seed germination and vigor parameters assessed under 10 °C. Treating the seeds with BABA and GABA at the rates of 0.50 and 2.00 mM, respectively, promote seed tolerance to cold stress and had positive effect on seed germination, emergence and vigor as well as the ROS activity.

Keywords: Carrot seeds, Seed treatment, Cold stress, BABA and GABA

3.1. Introduction

Cold stress is one of the most important and limiting environmental stress, and it can cause tremendous damage to the physiological and biochemical systems of the plants (CHEN et at., 2010). The germination process in order to proceed well needs a favorable condition of temperature, which can allow the metabolic reactions to occur, and for the most cultivated species the optimal temperature ranges from 20 – 30°C, been temperatures below 15°C considered extreme for many vegetables' species (MARCOS FILHO, 2012). According to Aghdam et al. (2012), the low temperature during germination and early seedling growth is considered one of the most factors that can reduce crop productivity.

Carrot (*Daucus carota*), when cultivated during the wintertime, is set to face serious problems related to seed germination, emergence, and early vegetative development. Mechanisms that can promote or help the plant to face this unfavorable condition and improve seed germination and emergence, uniformity and stand establishment, will be reflecting in yield increasing and profitability (PEREIRA et al., 2007; NASCIMENTO et al., 2013).

In order to face the diversity of growing conditions that very often are challenging plant growth as well as the high cost of breeding programs to produce varieties and hybrids for a certain stress tolerance, researchers have experimented and applied biostimulants to enhance crop stress tolerance (NAYYAR et al., 2014). By definition, biostimulants are substances based on microorganisms, microbial inoculants, humic acids, fulvic acids, protein hydrolysates, amino acids, and seaweed extracts to stimulate plant growth and development under unfavorable conditions. (SHARMA et al., 2014). Amino acids are one of those products that are essential for plant functioning mainly under stressful conditions where they act as antioxidants and signaling roles, contributing to the best development in unfavorable environmental conditions (SHARMA and DIETZ, 2005).

BABA (Beta-aminobutyric acid) and GABA (Gamma-aminobutyric acid) are amino acids that have been currently studied for many researchers to improve plant stress tolerance (NAYYAR et al., 2014; JISHA and PUTHUR, 2016). Malekzadeh et al. (2014), found out that the spray application of GABA (750 μM/L) on tomato seedlings (*Lycopersicon esculentum*) resulted in beneficial effects on reducing chilling stress by increasing growth of seedlings and antioxidant activity, and proline and soluble sugar content. Similar results were found by Nayyar et al. (2014) using GABA on nutrient solution (1 mM) in rice (*Oryza sativa*) under heat stress, resulting in growth improvement and survival rate, and increasing the antioxidant system. Zimmerli et al. (2008), on the other hand, studied the effect of BABA (0.5 mM) applied on nutrient solution in overcoming arabidopsis seedlings heat tolerance and concluded that this product increased the survival rate of the plants under this condition.

There are a few scientific works assessing the use of BABA and GABA as a seed treatment component, and among them, the use of these products as a priming component is the most studied. Jisha and Puthur (2016) studied the effect of seed priming with BABA on salt and osmotic stress tolerance of *Vigna radiata* seeds. Cheng et al. (2018) primed seeds of white clover with GABA under salt stress conditions, and in both studies, there were great results on seed stress tolerance.

Although the priming technic is efficient, it is very costly in time and resources in order to treat a certain volume of seeds. The film coating technic is a good way to apply plant protection products, biologicals and stimulants all within a thin film coat layer, reducing cost and products need, and positively impacting the environment. According to Bradácová et al. (2016) when a film coating is applied to the seed and there are on its products that can help in nutrient uptake and root growth, mitigating the effect of cold stress can be achieved. In the same way, Gómez-Muñoz et al. (2018) studied the effect of three seed treatments on cold stress tolerance of corn seeds and found out that the addition of Mn/Zn and inoculation with Penicillium reduced the adverse effects of cold stress.

With this, the aim of this study was to evaluate the effect of BABA and GABA on film coating seed treatment and its effect on cold stress tolerance of carrot seeds.

3.2. Material and methods

3.2.1. Seed treatment

The seeds of carrot (Daucus carot) cv. Amanda F1 were provided by Agristar do Brasil Inc., and was used for all studies. The film coating composition was made by applying per kg of seeds 20 $ml.Kg^{-1}$ of Maxim XL® (fungicide), 30 $ml.Kg^{-1}$ of Filmcat L083® (Incotec polymer), 30 $ml.Kg^{-1}$ of water and addition or not of amino acid in the following rates. The rates of BABA and GABA (Sigma Aldrich): 0.25, 0.50, 1.00 and 2.00 mmol L^{-1} were added to the film coating composition, seeds with polymer but no amino acid applied were used as the control. The formulations were applied using a laboratory scale conventional pan coater allowing a better uniform treatment application on coverage carrots seeds.

3.2.2. Effect of temperature on carrot seed germination

In order to assess temperatures, the cause heat stress, seeds of carrot were submitted to germinate under different temperatures: 10, 15, 20, 25, and 30ºC in BOD (Biochemical Oxygen Demand) chambers with light (12 h)/dark (12 h) photoperiod. Seeds were placed in light transparent plastic boxes (11 x 11 x 3.5 cm.) containing two sheets of blotting paper moistened with 14 ml of water. Each treatment was replicated four times and 50 seeds for each replicate. The germination rates were counted after 14 days according to the recommendations from the rules for seed testing (BRASIL, 2009) with results expressed as percentage of seed germinated.

3.2.3. Effect of BABA and GABA on seed germination and vigor under heat stress

Germination test. The treated and non-treated seeds with BABA and GABA were placed in light transparent plastic boxes (11 x 11 x 3.5 cm.) containing two sheets of blotting paper moistened with 14 ml of water and kept at 20 (ideal temperature) or 10°C (stress temperature) at 12 h light/dark photoperiod. The 35°C temperature was chosen based on the preliminary tested described at 2.2 item. Each treatment was replicated four times with 50 seeds for each replicate. The germination at the 7d (First count of germination), and 14 days (Total germination) were obtained according to the recommendations from the rules for seed testing (BRASIL, 2009).

Emergence of seedlings in greenhouse: The treated and non-treated seeds with BABA and GABA were sowed in four replications of 50 seeds in a multicellular expanded polystyrene tray containing the commercial substrate made from pine bark and placed in a greenhouse with 12 h light/dark photoperiod. Evaluations occurred after seven and 14 days of sowing, and the results were expressed as an average percentage of emerged seeds.

Field emergence of seedlings: Four replicates of 50 seeds for each treatment were sown in furrows 5.0 m long spaced 0.20 m apart and 0.01 m deep. Soil moisture was kept sufficiently wet by drip germination. The percentage of emerged seedlings was counted on the 21th day after sowing.

Germination and emergence speed index. The germination speed of the carrot seeds was determined based on the increase of the number of new seedlings counted daily throughout the test period. The following the formula was used (MAGUIRE, 1962):

GSI = (N1 / D1) + ... + (Nn / Dn); Where Nn = number of normal seedlings counted daily; Dn = days after test installation.

Dry weight of seedlings. At the end of the 14 days (germination and emergence test) and 21 days (field emergence test) samples of each replication were placed in an oven run at 80 ºC for 48 h in order to obtain the dry weight of seedling. The results were expressed in mg. seedling^{-1}.

3.2.4. Effect of BABA and GABA on antioxidant capacity of seeds under heat stress

Lipid peroxidation and H2O2 content. Treated seeds with BABA (0.50 g.Kg^{-1} of seeds) and GABA (2.00 g.Kg-1 of seeds), as well as the control treatment (0.00 g.Kg-1 of seeds) were collected at 6 days after sowing, frozen in liquid nitrogen and stored at -80 ºC for biochemical analysis. The rates were selected based on previous germination and vigor testes.

Lipid peroxidation was determined by measuring the content of Malondialdehyde (MDA), which was extracted with 0.25% thiobarbituric acid (TBA) in the absorbance at 532 and 600 nm and results expressed at mmol.g^{-1} of fresh matter (HEATH and PACKER, 1968). The H2O2 content was determined according to methodology described by Alexieva et al., (2001) with hydrogen peroxide (H2O2) standard curve. Seed material samples (0.1 g) were macerated in 0.1% (m/v) of trichloroacetic acid and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant (1 ml) was mixed with 1 mL of 100 mM potassium phosphate buffer (pH 7.5). The absorbance was read at 390 nm and the results expressed at $mmol.g^{-1}$ of fresh matter.

3.2.5. Statistical analysis

The statistical analysis of the results obtained in the evaluations were submitted to variance analyses by the F test. The treatment means were compared by the Tukey test ($p < 0.05$) using the R package stats (version 3.5.3) (R Core Team, 2019).

3.3. Results

3.3.1. Effect of temperature on carrot seed germination

The germination percentage as a function of different temperatures is presented in figure 1. The cold temperatures drastically affect seed germination percentage which decreased as a function of decreasing temperatures. Temperatures below 20 °C decreased gradually the germination percentage of carrot seeds from the cultivar Amanda F1, showing percentage of germination values of 79% when it was provided 20 °C to values of 54% (10º C) and even no germination at 5 ºC (0%). With this, the temperatures of 20 and 10 ºC were considered as optimal and stressed condition to carrots seed germination. According to the rules for seed testing and analysis the temperature of 20 °C is considered as an optimal test temperature (BRASIL, 2012).

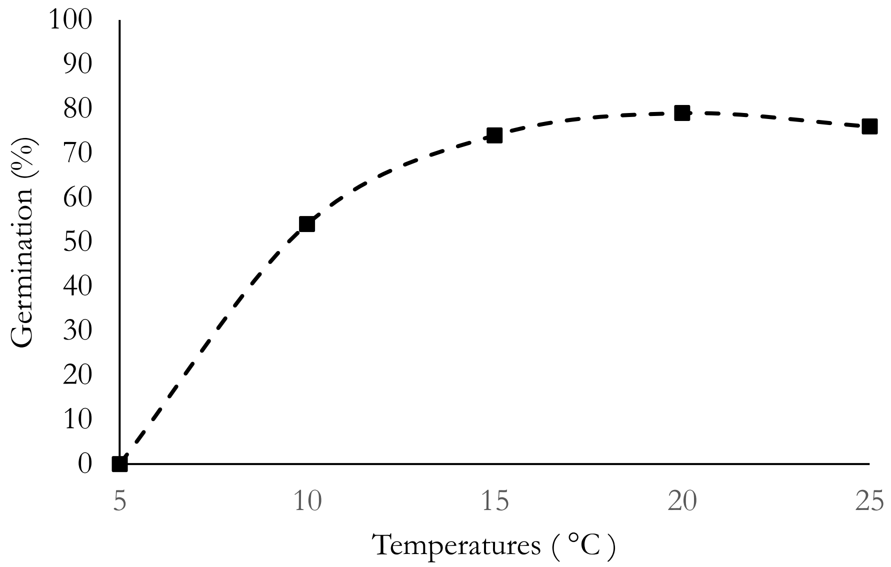

Fig. 1. Effect of different temperatures on carrot seed germination (Amanda F1)

3.3.2. Effect of BABA and GABA application on seed germination and emergence

The Table 1 shows the values of Germination percentage at 20 °C (GP20), Germination percentage at 10 °C (GP10), First-count germination at 20 °C (FG20), First-count germination at 10 °C (FG10), Thermotolerance (THT), Germination speed index at 20 °C (GS20), Germination speed index at 10 °C (GS10), Dry weight of seedlings at 20 °C (DW20), Dry weight of seedlings at 10 °C (DW10) for non-treated and treated seeds with BABA and GABA.

Table 1. Germination percentage at 20 °C (GP20) and 10 °C (GP35), First-count germination at 20 °C (FG20) and 10 °C (FG10), Germination speed index at 20 °C (GS20) and 10 °C (GS10), Thermotolerance (THT), Dry weight of seedlings at 20 °C (DW20), and 10 °C (DW10) of carrot seeds treated and non-treated with BABA and GABA

TRAT	GP20	GP10	FC20	FC10	THT	GIS20	GIS10	DW20	DW10
	%		%		T35/T20	Index		mg	
CK1	76 bc	52 c	68 bc	35 bc	0.68 c	7.59 ab	4.68 b	0.89 b	0.44 ns
CK2	76 bc	57 c	66 c	34 bc	0.75 c	7.69 ab	4.67 b	0.89 b	0.47 ns
B - 0.25	82 a	80 a	76 ab	46 ab	0.98 a	8.45 a	5.40 a	1.07 a	0.47 ns
B - 0.50	81 ab	76 ab	68 bc	52 a	0.94 a	7.60 ab	5.60 a	0.82 b	0.48 ns
B - 1.00	85 a	75 ab	80 a	53 a	0.88 ab	7.94 a	5.37 a	1.07 a	0.49 ns
B - 2.00	72 c	77 ab	62 c	32 c	1.07 a	6.72 b	5.42 a	1.02 a	0.51 ns
CV%	3.32	3.67	6.17	13.61	4.25	6.76	8.63	4.80	6.49
CK1	76 b	52 c	68 b	35 b	0.68 c	7.59 ns	4.68 ns	0.89 ab	0.43 ab
CK2	76 b	57 c	66 ab	34 bc	0.75 bc	7.69 ns	4.67 ns	0.89 ab	0.46 ab
G - 0.25	80 ab	70 ab	75 ab	31 bc	0.88 ab	7.69 ns	4.72 ns	0.93 a	0.43 ab
G - 0.50	79 ab	73 ab	68 ab	43 a	0.92 a	7.27 ns	5.27 ns	0.91 a	0.37 c
G -1.00	80 ab	75 a	74 ab	28 c	0.94 a	7.40 ns	4.85 ns	0.82 b	0.42 bc
G - 2.00	85 a	79 a	76 a	43 a	0.93 a	8.19 ns	5.16 ns	0.87 ab	0.47 a
C.V%	3.16	4.06	6.03	8.51	5.85	6.33	7.06	4.71	5.28

Means with the same letter within a column were not different by Tukey test ($p < 0.05$).

Germination percentage (GP): Under optimal condition for seed germination (20 °C), the seeds treated with BABA at the rates of 0.25, 0.50 and 1.00 mM performed better but were not statistically different between each other, showing the percentage of germination on the average of 82%. The rates of 2.00 mM, and the controls CK1 and CK2 had the lowest performance on seed germination. The germination percentage for seeds submitted to cold stress and treated with BABA was maintained to values similar to those obtained under optimal condition, while the control treatments had the germination significantly reduced. The rate of 0.25 mM showed the best results (80%), but did not differ from the other BABA rates. The control treatments had 52 and 57% of germination for the CK1 and CK2 respectively, been those values the lowest for the germination test. For GABA treatments under optimal condition, the seed germination was better performed when the seeds were treated with a rate of 2.00 mM, with the highest germination percentage (85%), but not statistically different from the rates of 0.25, 0.50, and 1.00 mM. The controls CK1 and CK2 showed the lowest germination percentage but were not different from the 0.25, 0.50, and 1.00 mM rates. Under cold stress, the seeds treated with GABA also maintained the percentage of germination similar to those values from the optimal condition, with the rates of 1.00 and 2.00 mM showing the highest germination percentage, 75 and 79%, respectively, but not differing from the other GABA rates. The control treatments CK1 and CK2 were responsible for the lowest results.

First count of germination (FC): The first count of germination (FC) for seeds treated with BABA under 20°C at the rate of 1.00 mM was responsible for the best results but did not differ statistically from the 0.25 mM rate. The control treatments CK1 and CK2, 0.50 and 2.00 mM were responsible for the lowest results of the first count of germination. Under cold stress conditions, the rates of 0.50 and 1.00 mM presented the best results but did not differ from the 0.25 mM. The control treatments and the rate of 2.00 mM presented the lowest results for this parameter. The seeds treated with GABA under the optimal condition at the rates of 0.25, 1.00 and 2.00 mM present the best results for the first count of germination, and did not differ from each other. Under cold stress temperature, the rates of 0.50 and 2.00 mM presented the best results. The lowest values of the first count of germination were presented from the rate 1.00 mM, but did not differ from the control CK2 and the rate of 0.25.

The *Thermotolerance* (THT) of seeds to germinate under cold temperature was significantly improved by the treatments with BABA and GABA as compared to the controls. BABA treatments in general improved THT for carrot seeds germinate. The best results were obtained when the rates of 0.25, 0.50 and 2.00 mM were provided, but the rate of 1.00 mM did not differ from the others. The control treatments CK1 and CK2 had the lowest values for THT. In a similar way, GABA improved the thermotolerance with all the rates tested as compared to the control treatments. The rates of 0.50, 1.00 and 2.00 mM were responsible for the best results, but 0.25 mM did not differ from them.

The *Germination speed index* (GSI) under the optimal condition for BABA treatments at the rates of 0.25 and 1.00 mM presented the best results but did not differ from the controls CK1 and CK2, and the rate of 0.50 mM. The rate of 2.00 mM presented the lowest values for GSI. Under cold stress conditions, however, the BABA treatments improved the GSI with all the rates assessed, and the controls CK1 and CK2 presented the lowest results. GABA treatments showed no influence on the GSI either for the optimal condition or for the cold stress and the rates assessed did not differ from the controls in any condition analyzed.

The *Dry weight of seedlings* (DW) under optimal condition was positively improved by BABA treatments and the rates of 0.25, 1.00 and 2.00 mM presented the best results. The control treatments and the rate of 0.50 had the lowest values for this parameter. Under cold stress, there was no statistical difference between the BABA and the control treatments. The GABA treatment at the rates of 0.25 and 0.50 mM presented the best results but did not differ from the controls CK1 and CK2 as well as the rate of 2.00 mM. The rate of 1.00 was responsible for the lowest dry weight of seedlings. Under cold stress conditions, the rate of 2.00 mM presented the highest dry weight of seedlings but did not differ from the rates of 0.25 and the controls CK1 and CK2. The rates of 0.50 and 1.00 presented the lowest values for this variable.

The results of carrot seed field emergence (FE), greenhouse emergence (E), greenhouse first count of emergence (FCE), emergence speed index (ESI), and dry weight of seedlings of the emergence test are presented in table 2.

Table 2. Field emergence (FE), Greenhouse emergence (E), First count of greenhouse emergence (FCE), Emergence speed index (GSIE) and Dry weight of seedlings of carrot seeds treated and non-treated with BABA and GABA

TRAT	FE	E	FCE	ESI	DWE
		%		*Index*	*mg*
CK1	36 c	62 b	54 b	3.08 ns	30.33 bc
CK2	43 bc	67 b	52 b	3.52 ns	28.82 c
B - 0.25	51 ab	75 a	62 a	3.65 ns	27.54 c
B - 0.50	53 a	79 a	64 a	3.85 ns	33.09 ab
B - 1.00	53 a	74 a	64 a	4.12 ns	34.80 ab
B - 2.00	55 a	72 a	67 a	4.00 ns	37.45 a
CV%	8.15	5.95	10.86	7.60	7.61
CK1	36 c	62 b	54 b	3.08 ns	30.33 b
CK2	43 bc	67 b	52 b	3.52 ns	28.82 c
G - 0.25	58 a	72 a	63 a	3.59 ns	36.92 ab
G - 0.50	53 ab	74 a	65 a	4.05 ns	35.42 ab
G -1.00	55 a	75 a	68 a	4.32 ns	37.70 ab
G - 2.00	56 a	78 a	67 a	3.58 ns	42.49 a
C.V%	7.84	4.14	10.42	9.18	15.39

Means with the same letter within a column were not different by Tukey test ($p < 0.05$).

The field emergence was improved by the film coating treatments with BABA and GABA as compared to the control treatments CK1 and CK2. The field emergence was increased in the average of 18% when the seeds were film coated with BABA at the rates of 0.50, 1.00 and 2.00 mM as compared to the CK1 control, but did not differ from the rate of 0.25 mM. The control treatments were responsible for the lowest field emergence percentages. GABA treatments presented a similar effect on the field emergence, and the rates of 0.25, 1.00 and 2.00 mM significantly increased field emergence in the average of 20% as compared to the control CK1 treatment, but did not differ from the rate of 0.50 mM. The control treatments were responsible for the lowest results of field emergence.

The *greenhouse emergence* was positively affected by the film coating treatment with BABA and GABA. It was observed that the treatments with BABA regardless of the rate analyzed it had a positive effect on the emergence of carrot seedlings in the greenhouse conditions, increasing on average of 13% as compared to the control CK1. For the GABA treatment, all the rates assessed had a positive effect on carrot greenhouse emergence, increasing on average 12.75% as compared to the CK1 control. The control treatments CK1 and CK2 was responsible for the lowest results when it is compared to both amino acids.

BABA and GABA seed treatments had a positive effect on the *First count of emergence*. Regardless of the rate tested, the treatments that had BABA were superior to the controls Ck1 and CK2 treatments, increasing the first count of emergence in the average of 10.5% as compared to the CK1 control. In the same way, treatments that had GABA on its composition showed the best performance for the first count of emergence as compared to the control CK1 and CK2, regardless of the rate tested, increasing in the average of 11.75% as compared to the control CK1 treatment.

The *greenhouse emergence speed index* did not show statistical difference among the treatment regardless of BABA, GABA and the controls CK1 and CK2.

The *Dry weight of seedlings* was significantly improved by the treatments that had BABA and GABA on its composition. For the BABA treatments, the rate of 2.00 mM was responsible for the best results and increased the DWE in 7.12 mg as compared to the control CK1. The mentioned rate did not differ statistically from the rates of 0.50 and 1.00 mM. The control treatments CK1, CK2 and the 0.25 mM rate were responsible for the lowest dry weight of seedlings. GABA treatments also improved the dry weight of seedlings. The rate of 2.00 mM showed the best results for this parameter and increased the DWE in 12.16 mg, but did not differ from the other GABA rates assessed. The control treatments CK1 and CK2 were responsible for the lowest values for DWE.

3.3.3. Effect of BABA and GABA application on antioxidant capacity of seeds under cold stress

The Hydrogen peroxide content in carrot seeds is presented in figure 3. did not have a statistical difference between the treatments analyzed, showing similar values under cold stress and optimal condition. For the MDA content, it was observed that under optimal conditions the Control treatment (0.200) increased the MDA content in 2.5 and 1.92 times when it is compared to as compared to treatments that received BABA (0.080) and GABA (0.104), respectively. When seeds were submitted to cold stress there was an increase in MDA content for all treatments, but the treatment with GABA (0.164) showed the best results and was statistically different from the Control (0.204) and BABA (0.199), reducing with this the deleterious effect of MDA content on carrot seeds under cold stress condition.

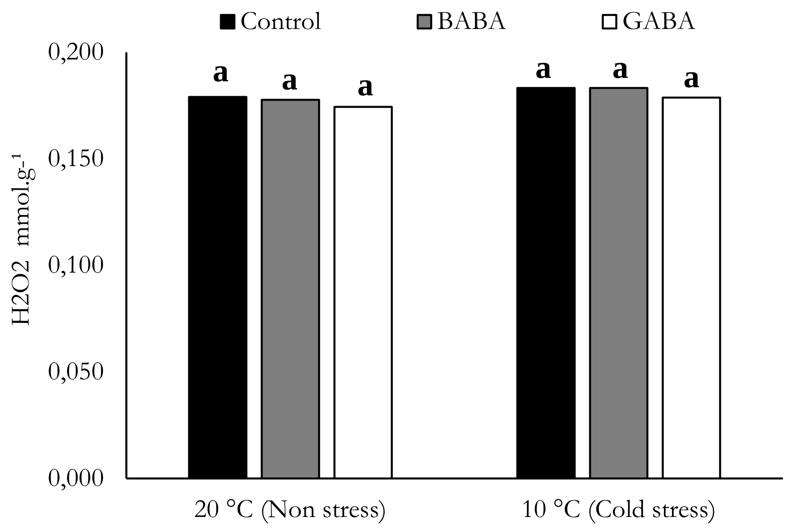

Fig. 2. Hydrogen peroxide (H_2O_2) in carrot seeds after treatment with GABA, BABA and control under optimal condition (20 °C) and heat stress (10 °C).

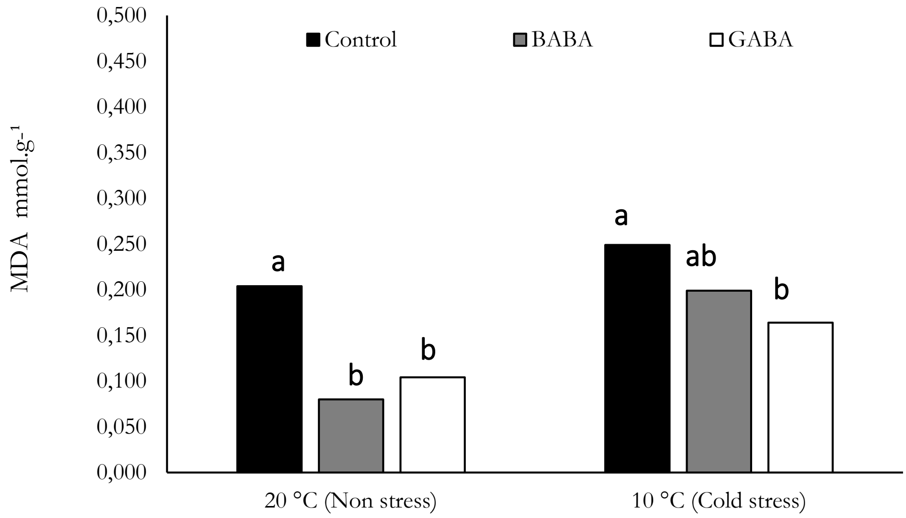

Fig. 3. Lipid Peroxidation (MDA) in carrot seeds after treatment with GABA, BABA and control under optimal condition (20 °C) and cold stress (10 °C).

3.4. Discussion

Temperature plays an important role in carrot seed germination, with a significant influence on seedling emergence and development (NASCIMENTO et al., 2008; NASCIMENTO et al., 2013). The optimal range of temperature for carrots seeds to germinate varies between 20 and 30 ºC and bellow this condition, the seed germination declines gradually. Studies involving the effect of cold stress on carrot seeds germination and early development are scarce in the scientific literature, and the main researches are assessing the effect of heat stress, even though the cold stress can significantly reduce carrot seed germination and seed development. In our results, we could find that cold stress had negative effect on the percentage of germination, first count of germination, germination speed index, and dry weight of seedlings.

The seed treatment using BABA and GABA significantly improved seed germination and vigor under the cold stress conditions, and made seeds to tolerate the disadvantage of the temperature and have similar behavior than in the optimal condition. Similar results were found by Yadav et al. (2011) when studied the effect of seed priming on *Capsicum* seeds germination under temperature of 15 °C. By treating corn seeds with *Penicillium sp.* or Mn/Zn added to the film coating treatment, Gómez-Muñoz et al. (2018) found that those treatments alleviated the negative effects of cold stress. However, studies involving the use of film coating with the addition of amino acids are not very diffused in the carrot seed treatment literature, been necessary to study the suitable rate to treat the seeds in order to obtain the best results provided from the amino acids.

Many researchers have studied the application of BABA and GABA in varius different ways, but the foliar application has been the most applied. The film-coated treatment added amino acid is a very efficient method since it can be applied during the standard procedure that seed companies usually do on their seeds and the required rates to promote the best effects are small. BABA and GABA are efficient amino acids with a role in the antioxidant system of plants, with this promoting plant stress tolerance under environmental stresses as salt, hydric, cold and heat. Researches analyzing the effect of BABA or GABA in cold stress are not very common in the literature, and BABA has been the less used. Jisha and Puthur (2016) recommended lower rates of BABA on seed treatment in order to obtain the best results since higher rates can let to a reduction in seedling growth and development. For GABA application, Cheng et al. (2018) recommended rates varying from 0.5 to 2.5 mmol.L-1 on seed priming for white clover seeds under salt stress conditions, and as the same way then Jisha and Puthur (2016), the author found that rates above 5 mmol.L-1 can cause inhibited effect on seed germination and vigor. The results found in the literature are aligned with this study, where rates of BABA at 0.50 mM and GABA 2.00 mM were responsible for the best results on germination and vigor of carrot seeds submitted to cold stress condition.

The thermotolerance is a measurement obtained by the ratio between the germination percentages values from cold stress (10 °C) temperatures by those from optimal conditions (20°C) (NASCIMENTO et al., 2008). This information shows the ability of certain cultivar to germinate under low temperature conditions, as the ration increases the thermotolerance increases as well. The control treatments had a thermotolerance ratio of 0.30 in this study, and even though the variety used can be considered a tolerant hybrid to high temperatures, the thermotolerance ratio is very low when it is compared to results obtained by Nascimento et al. (2008) when analyzed more than 60 genotypes, and obtained values above 0.60 for the open pollinated varieties Brasilia, Esplanada and Alvorada. The use of BABA and GABA as a component of film coating allowed seeds to get more tolerant to higher temperatures, and increased thermotolerance values to above 0.60.

In the same way that in the high temperatures, the low-temperature conditions can cause a negative effect on plant growth and development, significantly impacting seed germination, speed of germination and seedling growth. Plants have adapted to tolerate cold environments by making some adjustments on physiological apparatus to support the reactions and compounds that are excessively produced during this period. ROS species are the main compounds produced excessively in stress conditions, regardless of the type of stress submitted. Excessive accumulation of ROS and lipid peroxidation in plants and an efficient antioxidant system against those compounds can guarantee the success of the seed germination process (BAILLY, 2004; CHEN et al., 2010).

It has been scientifically proved that BABA and GABA are efficient in improving antioxidant enzyme activities and with this reducing the oxidative damage caused by excessively produced ROS components. Under stress conditions, the generation of ROS leads to membrane damage and lipid peroxidation, resulting in an excessive increase of MDA content (MITTLER et al. 2004; CARILLO, 2018).

The relatively high content of MDA in carrot seedlings from the control treatment and its abrupt increment when submitted to the cold stress conditions reveals that lipid peroxidation increase as cause of increased ROS generation, and it was observed that by treating seeds with BABA and GABA the MDA content was significantly reduced as compared with the control, with more effect on the GABA treatment as compared to them both. Malekzadeh et al. (2014), studying foliar application of GABA on promoting cold stress tolerance to tomato seedlings (*Lycopersicon esculentum*) found that it significantly improved cell membrane stability, delayed leaf senescence, and enhanced the osmotic adjustment, but more than that, it reduced the MDA content and with this can be considered one of the main reason for alleviating the damaging effect of cold stress. Jisha and Puthur (2016), even though they did not assess the effect of cold stress, found out a reduction in the MDA content under NaCl and Osmotic stress in *Vignia radiata* seeds primed with BABA.

3.5. Conclusion

Cold stress temperatures drastically reduced carrot seed germination, vigor and increased MDA content. The application of BABA and GABA as a component of a film coating treatment at the rate of 1.00 and 2.00 mM for the respective amino acids resulted in increasing seed germination, emergence, and vigor. The amino acids also reduced the MDA activity under cold stress conditions.

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