

Inducing flowering in *Noccaea caerulescens* (J. & C. Presl) F. K. Mey (Brassicaceae), a species having high heavy-metal accumulation¹

Indução ao florescimento de *Noccaea caerulescens* (J. Presl & C. Presl) F. K. Mey (Brassicaceae), uma espécie hiperacumuladora de metais pesados

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ABSTRACT - *Noccaea caerulescens* (J. & C. Presl) F. K. Mey (Brassicaceae) is a plant widely used in studies of the high accumulation of such metals as Cd, Ni, and Zn. Considered a biennial species, propagated mainly by seed, it presents a long period for the generation of new individuals, which significantly hampers the development of new studies of this species. Based on the above, the objective of this work was to develop a protocol to induce early flowering and abundant seed-production in *N. caerulescens*. Plants of this species were therefore grown and developed in a controlled environment (growth room and cold chamber), and then submitted to different treatments composed of combinations of the following environmental conditions: photoperiod (8 or 12 hour of light - 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$), temperature (4 or 21 °C), time of exposure to the above conditions (6 or 8 weeks) and application of different concentrations of GA₃ (0, 15, 30 and 60 $\mu\text{g } \mu\text{L}^{-1}$). The work was carried out in a completely randomized design, with twenty plants per treatment being analyzed. The following characteristics were evaluated: a) Percentage of flowering (%); b) Length of main cluster (cm); and c) seed production (g pl^{-1}). It can be seen that the temperature of 4°C was essential for inducing flowering and seed-production in *N. caerulescens*, with the time of exposure of the plants to this temperature being very important to potentialise seed production and longer periods of exposure to low temperatures being preferred.

Key words: *Thlaspi caerulescens* (J. & C. Presl). GA₃. Main cluster. Seed production. Vernalization.

RESUMO - *Noccaea caerulescens* (J. & C. Presl) F. K. Mey (Brassicaceae) é uma planta muito utilizada em estudos de hiperacumulação de metais como Cd, Ni e Zn. Considerada espécie bienal, sendo propagada basicamente por sementes, apresenta longo período de tempo para a geração de novos indivíduos, o que prejudica consideravelmente o desenvolvimento de novos estudos com esta espécie. Baseado no exposto objetivou-se neste trabalho, o desenvolvimento de um protocolo para indução de florescimento precoce e abundante de *N. caerulescens*. Para isso, plantas desta espécie foram crescidas e desenvolvidas em ambiente controlado (sala de crescimento e câmara fria), sendo então submetidas a diferentes tratamentos que eram compostos por combinações de diferentes condições ambientais: fotoperíodo (8 ou 12 horas de luz - 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$), temperatura (4 ou 21 °C), semanas de exposição às condições de luz e temperatura citadas anteriormente (6 ou 8 semanas) e aplicação de concentrações de GA₃ (0; 15; 30 e 60 $\mu\text{g } \mu\text{L}^{-1}$). O trabalho foi conduzido em delineamento inteiramente casualizado, sendo analisadas vinte plantas por tratamento. As seguintes características foram avaliadas: a) Percentual de florescimento (%); b) Comprimento do pendão principal (cm); e c) Produção de sementes (g pl^{-1}). Pode-se observar que a temperatura de 4 °C foi essencial para a indução ao florescimento e produção de sementes de *N. caerulescens*, sendo que o tempo de exposição das plantas a esta, foi muito importante para a potencialização da produção de sementes, sendo desejados períodos mais longos de exposição à baixa temperatura.

Palavras-chave: *Thlaspi caerulescens* (J. & C. Presl). GA₃. Pendão principal. Produção de sementes. Vernalização.

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INTRODUCTION

All plant species monitor the environment and their own internal signals in order to ensure their reproduction at the most appropriate time of the year and correct stage of development (BALASUBRAMANIAN *et al.*, 2006; BOUVERET *et al.*, 2006; COBBETT, 2003, PRASAD, FREITAS, 2003). Thus, the propagation and survival of a plant species depends critically on its ability to regulate with accuracy its phases of transition between vegetative and reproductive growth (KOMEDA, 2004; YANG *et al.*, 2006).

Researchers have been able to bring forward the flowering of certain species, such as *A. thaliana*, *T. arvense* and *Brassica napus*, belonging to the same family as *N. caerulescens* (Brassicaceae), by modifying the photoperiod (DAHANAYAKE; GALWEY, 1999), the temperature (BALASUBRAMANIAN *et al.*, 2006) and the application of gibberellin (DAHANAYAKE; GALWEY, 1999).

Several studies have been conducted using the species *Arabidopsis thaliana*, with the aim of establishing flowering pathways (AUSIN; ALONSO-BLANCO, MARTINES-ZAPATER, 2005; BERNIER; PÉRILLEUX, 2005; QUESADA, DEAN; SIMPSON, 2005). However, for other plant species, such as *Noccaea caerulescens*, little information is available about simulating the best conditions for producing these events.

N. caerulescens also known as *Thlaspi caerulescens* (UNIPROT TAXONOMY) is considered a compact plant (PEER *et al.*, 2003). Its leaves present an opposite arrangement, with obtuse distal and attenuated proximal parts. Its flowers are small, whitish in colour and self-pollinating (BESNARD *et al.*, 2009). Cross-fertilization may also occur between different compatible populations (BASIC; BESNARD, 2006). The fruits are in the shape of silica, with dehiscence being seen from six to eight weeks after anthesis. Generally three to five seeds are seen per fruit. *N. caerulescens* is not grown commercially, being found in nature in many temperate regions (BESNARD *et al.*, 2009). In recent years it has received special attention due to having been selected as a model system for molecular-genetic studies of the over-accumulation of metals by plants (PEER *et al.*, 2003). According to several researchers, this species is able to hyperaccumulate such metals as Cd, Ni and Zn (GUIMARÃES; GUSTIN; SALT, 2009; NASCIMENTO; XING, 2006). However, it has the great disadvantage of having a long life cycle (biennial plant; PEER *et al.*, 2003), requiring two cold seasons, one for breaking seed dormancy, and the second for vernalisation, to promote flowering.

In the case of photoperiod-induced flowering, it is known to occur in the species *A. thaliana* by means

of signals over long distances between the leaves and the apical meristem, via the phloem (BERNIER; PÉRILLEUX, 2005).

However, many temperate species (as is the case of *N. caerulescens*), only bloom after they have gone through a prolonged cold period, a process known as vernalisation, which allows for the 'alignment' of the induction of flowering and those climate conditions which are favourable for its proper development (HENDERSON; DEAN, 2004). In general, winter annuals are vernalized when at the seedling stage, while biennials require a long period of development at low temperatures in order to stimulate flowering.

According to Yamaguchi and Kamiya (2000) the environmental factors, photoperiod and temperature, can alter the levels of active gibberellins, affecting gene transcription at specific stages in the biosynthetic pathways of this hormone. The gibberellins take part in the regulation of several physiological processes, including the germination of seeds, establishing of fruit and development of flowers. However, the application of gibberellins may cause a decrease in the expression of those genes which encode the synthesis of such active gibberellins as GA₂₀ oxidase and GA₃ oxidase, and on the contrary, result in an increase in transcription of the GA₂ oxidase degradation genes (ELLIOTT *et al.*, 2001) so inhibiting flowering. The enzymes, GA₂₀ oxidase GA₃oxidase, directly participate in the formation pathways of the GA_{1/4} active gibberellins (TAIZ; ZEIGER, 2004). According Taiz and Zeiger (2004), GA₃ can replace GA₁ in most bioassays.

Given the above, the objective of this work was to develop a protocol for inducing early and abundant flowering in *N. caerulescens*.

MATERIAL AND METHODS

Plant Material

The original seeds of the hyperaccumulating plant, *N. caerulescens* St. Félix de Pallières, came from France, as described by Peer *et al.* (2003).

Germination

Twenty-five seeds of *N. caerulescens* were germinated on plates (100 x 15 mm) containing 0.5 x Murashige and Skoog medium (MSP001, CAISSON Laboratories, Inc. / www.caissonlabs.com) with the further addition of vitamins (MSV11001, CAISSON Laboratories, Inc.), 1.2% agar (ACUMEDIA manufacturers, Inc.) and 3.0 mg l⁻¹ Benomyl (methyl 1-(butilcarbomil)-2-benzimidazolcarbomato (SIGMA, St. Louis, MO)). After

seeding, the plates were incubated at 4 °C for 5 days, and following this period of seed stratification, were placed vertically in an incubator under controlled conditions (16 hours light: 8 hours darkness, with temperatures ranging from 18-23 °C). Germination occurred approximately one week after transfer.

Growing conditions

The experiment was conducted in plant growth chambers and cold rooms located on the campus of Purdue University, in West Lafayette, Indiana, USA. Seedlings at 20 days were individually transferred to 0.3L pots filled with substrate (Premier Promix PGX, Canada). The plants were irrigated twice a week with a nutrient solution containing 200 mg L⁻¹ N, 29 mg L⁻¹ P, 167 mg L⁻¹ K, 67 mg L⁻¹ Ca and 30 mg L⁻¹ Mg, and supplied with micronutrients using a commercial formula (MiracleGro® Excel® 15-05-15 Cal-Mag; The Scotts Co, Marysville, Ohio). The pH of the nutrient solution was adjusted to 5.7 to 6.0, using 0.08 ml of 93% sulphuric acid (v/v, Ulrich Chemical, Indianapolis) per litre of solution (ASCHENBECK, EDDY, 2004). The plants were kept in a greenhouse for 8 weeks under controlled environmental conditions (12 hours light (200 μmol m⁻² s⁻¹): 12 hours darkness, with temperatures ranging from 19 to 25 °C).

Treatments

The treatments were: amount of light in hours (8 or 12 hours (100 μmol m⁻² s⁻¹), temperature (4 or 21 °C), weeks of exposure to the above conditions of light and temperature (6 or 8 weeks), and application of different concentrations of GA₃ (0, 15, 30 and 60 μG μL⁻¹). One μL of the GA₃ solutions (0, 15, 30 or 60 μG μL⁻¹ GA₃ in 10% v/v water/acetone, with the addition of 0.05% Tween 20) were applied at the apex of the plant. There were ten applications of each solution, which were carried out on alternate days. After a period of 6 or 8 weeks of exposure to the above light and temperature conditions, the plants were transferred to new growth conditions (21 °C, 12 hours light, 200 μmol m⁻² s⁻¹), where they remained for around 6 to 8 weeks, that is until the fruits were completely ripe. After this period, plant irrigation was stopped in order to facilitate harvesting and quantification of the seeds.

Evaluated characteristics

Flowering was evaluated at 0 and 50 days after transfer (DAT) of the plants, for the treatment conditions of 21 °C, 12 hours light, 200 μmol m⁻² s⁻¹, by assessing the start of flowering at both the apex and central part of the plants. The height of the main tassel was determined by measuring the length from the base of the main stem to

its highest point. The mass of seeds was collected about 2 weeks after stopping irrigation, when their amount was determined by collecting the seeds from each individual plant into paper bags. The seeds were separated from the other parts of the plants (stems, leaves, pods, etc) and weighed in grams per plant.

Statistics

The study was carried out employing a completely randomised design in a 2 x 2 x 2 x 4 factorial scheme: two values for the amount of light in hours (8 and 12 hours of light (100 μmol m⁻² s⁻¹); two temperature conditions (4 and 21 °C); two values for weeks of exposure to the above conditions of light and temperature (6 and 8 weeks); and the application of four different concentrations of GA₃ (0, 15, 30 and 60 μG μL⁻¹). Twenty plants were evaluated for each treatment.

To evaluate flowering, the twenty plants from each treatment were divided into four lots of five plants, with the percentage of flowering plants being determined as the average percentage of the four lots.

The data were statistically analysed and tested for significance (p≤0.05). In order to determine the difference between treatments, the Scott-Knott test in the system for statistical analysis - SAEG (RIBEIRO JUNIOR, 2001) was used.

RESULTS AND DISCUSSION

Relationship between the different treatments and flowering

Before the transfer of those plants which had been under different environmental conditions for growth and development (8 or 12 hours of light per day, 4 or 21 °C, and 6 or 8 weeks exposure), to the final environmental growing conditions (21 °C, 12 hours light, 200 μmol m⁻² s⁻¹), it was found that in the treatments with low temperatures (lt, 4 °C), regardless of the exposure time, length of day and/or concentration of applied GA₃, the plants showed a higher percentage of flowering compared to those treatments conducted without low temperatures (wlt, 21 °C, table 1). These results differ from those presented by Peer *et al.* (2003), who found percentages for flowering of between 90 and 100%, only when plants of *N. ccaerulescens* were subjected to conditions of 4 °C and 8 hours of light for a period of 10 weeks. These results suggest that vernalisation is the main controlling factor of floral induction in *N. caerulescens*, and that gibberellin is not a substitute for the low temperatures required to obtain the maximum rates for flowering.

Table 1 - Percentage of flowering (%) in plants of *N. caerulea* under different periods of daily exposure to light (8 and 12 hours), temperatures (4 and 21°C), weeks of exposure to the above-mentioned combinations of light and temperature, and to different concentrations of gibberalin (GA) applied to the apical growth zone. Purdue University, USA, 2009

	Light (hours)	Temp. (°C)	Time (weeks)	Percentage flowering (%)			
				GA ($\mu\text{g } \mu\text{L}^{-1}$)			
				0	15	30	60
0 DAT*	8	4	6	100 a	100 a	100 a	100 a
	8	4	8	100 a	100 a	100 a	100 a
	12	4	6	100 a	100 a	100 a	100 a
	12	4	8	100 a	100 a	100 a	100 a
	12	21	6	0	5 b	0	0
	12	21	8	0	10 b	20 b	10 b
50 DAT*	8	4	6	100 a	100 a	100 a	100 a
	8	4	8	100 a	100 a	100 a	100 a
	12	4	6	100 a	100 a	100 a	100 a
	12	4	8	100 a	100 a	100 a	100 a
	12	21	6	0	10 b	18 b	12 b
	12	21	8	0	22 b	28 b	19 b

*0 and 50 DAT - 0 and 50 days after transfer of the plants from different treatments to the same conditions of temperature (21°C) and length of day (12 hours of light, $200 \mu\text{mol.m}^{-2} \text{s}^{-1}$). **Different letters in the columns indicate a statistical difference between treatments at a 5% level of significance, according to the Scott-Knott test. The Coefficient of Variation (C.V.) for flowering percentage - 13,22%

At 50 days after transfer (50 DAT) of the plants a result similar to that observed previously was seen, however, although not statistically significant, there was a slight increase in the flowering percentage for those treatments that had not been subjected to a temperature of 4 °C. This may indicate that the requirement for vernalisation is reduced based on the age of the plant, since the increase in internal gibberellin levels may play a more prominent role (Table 1).

The length of day did not affect the flowering percentage in plants at 4 °C, since both plants receiving 8 hours of light and those receiving 12 hours, presented 100% flowering (Table 1). However, when at the higher temperature of 21 °C, a greater flowering percentage was seen at both 0 and 50 DAT in those plants under long-day conditions, and which received applications of GA_3 , (7.5 and 18.2% on average respectively). These results are in contrast to those presented by Dahanayake and Galwey (1999), who by the application of 10 or 40 $\mu\text{g } \mu\text{L}^{-1}$ GA_3 at the apex of plants of *Brassica napus*, were able to produce the effect of low temperatures and greater daylight time (12 hours) on the flowering of this species. For *N. caerulea*, in the absence of low temperatures (4 °C), the application of GA_3 together with the largest photoperiod under study (12 hours of

light) appears to promote at least partial flowering, since when they were not available, no flowering plants were observed (Table 1). GA_3 therefore was not able to replace the demand for longer photoperiods (12 hours) and/or low temperatures (4 °C) in the flowering of this species.

One justification for the poor, or absence of, flowering in *N. caerulea* when not under low-temperature conditions (4 °C) for any length of time, may be related to the expression of the gene, FLOWERING LOCUS C (FLC). This gene is strongly expressed in the non-vernalised apical meristem of the stalk in plants of *A. thaliana*, which require a period of cold for flowering (MICHAELS; AMASINO, 2000). Several researchers have found that this gene acts as a repressor of flowering (AMASINO, 2004; D'ALOIA; TOCQUIN; PÉRILLEUX, 2008; RATCLIFFE *et al.*, 2003), and after vernalization, the gene appears to be turned off due to the deacetylation of the chromatin resulting in a modification of its structure (AMASINO, 2004). According to Burn *et al.* (1993) and Finnegan *et al.* (1998), methylation of the DNA also causes late flowering, whereas vernalization in such plants as *Arabidopsis* and *T. arvense* was effective in promoting demethylation in these species, and encourage flowering.

Relationship between the different treatments and the height of the main tassel

At 0 DAT, it can be seen that those plants under treatments conducted at a temperature of 21 °C showed the greatest height for the main tassel (8.5 cm on average, Table 2) when compared to plants at a temperature of 4 °C (2.1 cm on average). This result can be attributed mainly to the fact that at a temperature of 21 °C the metabolic activity of the plants remains high, with no change in the process of cell division and differentiation, nor in the assimilation and transformation of atmospheric CO₂ into carbohydrates within the plants, thereby affording their continuous growth and development. This was not observed in plants at a temperature of 4 °C, mainly due to a reduction in metabolic activity caused by low temperatures, which changes the optimal configuration for the action of those enzymes present in various metabolic processes of the plant. This change culminates in a reduction of photosynthetic activity and cell division, which causes a decrease in the rate of growth and development of the plants (BUCHANAN, GRUISSEM, JONES, 2001; TAIZ; ZEIGER, 2004).

However, at 50 DAT, all the treatments at a temperature of 4 °C showed greater height for the main

tassel (34.8 cm on average) than did plants which were kept at a temperature of 21 °C (11.1 cm on average). Metzger (1985) obtained similar results for *T. arvense*, finding that prolonged treatments at 4 °C were necessary to stimulate growth of the main tassel in that species. According to Hazebroek and Metzger (1990), in the absence of cold there is a large accumulation of entkaurenoic acid at the stem apices, the area where the stimulus to cold is perceived. After this treatment, and a return to high temperatures, the entkaurenoic acid is converted to gibberellin which, according to Taiz and Zeiger (2004), stimulates cell elongation and division. Such information allows the supposition that the greater size of the main tassel, seen in plants of *N. caerulea* at 4 °C when compared to those at 21 °C, may be due primarily to the presence of higher endogenous concentrations of gibberellins in their stem apices.

In table 2, it can be noted that the application of GA₃ associated with 12 hours of light at 21 °C, has an influence on the height of the main tassel of the plants at 0 DAT, with a greater average height for the main tassel (12.4 cm) being recorded in plants under these conditions. Plants receiving GA₃ application, under conditions of 8 hours light and temperatures of 21 °C, reached an average height of 7.3 cm, and those under conditions of 8 or 12

Table 2 - Average length of the main tassel in plants of *N. caerulea* under different periods of daily exposure to light (8 and 12 hours), temperatures (4 and 21°C), weeks of exposure to the above-mentioned combinations of light and temperature, and to different concentrations of gibberellin (GA) applied to the apical growth zone. Purdue University, USA, 2009

	Light (hours)	Temp. (°C)	Time (weeks)	Length of main tassel (cm)			
				GA (µg µL ⁻¹)			
				0	15	30	60
0 DAT*	8	4	6	1.59 d	2.07 e	2.02 d	2.05 e
	8	4	8	1.35 d	2.01 e	2.11 d	2.10 e
	12	4	6	3.57 c	7.09 d	7.12 c	5.71 d
	12	4	8	3.07 c	8.50 c	7.46 c	8.01 c
	12	21	6	4.24 b	10.22 b	11.56 b	11.94 b
	12	21	8	6.38 a	12.83 a	14.07 a	13.57 a
50 DAT*	8	4	6	31.17 a	34.30 a	35.04 a	36.08 a
	8	4	8	28.01 a	35.61 a	35.47 a	38.10 a
	12	4	6	5.18 b	9.75 d	9.83 c	8.88 d
	12	4	8	4.00 b	12.80 c	10.03 c	11.97 c
	12	21	6	6.14 b	14.28 b	16.27 b	16.62 b
	12	21	8	7.33 b	14.11 b	15.73 b	15.12 b

*0 and 50 DAT - 0 and 50 days after transfer of the plants from different treatments to the same conditions of temperature (21°C) and length of day (12 hours of light, 200 µmol.m⁻² s⁻¹). **Different letters in the columns indicate a statistical difference between treatments at a 5% level of significance, according to the Scott-Knott test. The Coefficient of Variation (C.V.) for flowering percentage - 11,22%

hours light at 4 °C, with or without GA₃, reached an average height of 2.1 cm.

In contrast to those seen at 0 DAT, at 50 DAT (Table 2), the greatest heights for the main tassel were recorded in those treatments at 4 °C (34.8 cm on average), thereby highlighting the greater heights for the main tassel in treatments with the application of GA₃, compared to treatments without GA₃ (36.1 to 30.6 cm on average respectively).

At 50 DAT, the greater height for the main tassel, which was observed in the treatments at 4 °C, was mainly due to the flowering of the plants of *N. caerulea*, which, after being subjected to lt, possibly had their endogenous concentrations of gibberellin increased, stimulating cell elongation and division as well as the transition from the juvenile to the mature stage of the plant (TAIZ; ZEIGER, 2004). Such results are an indication that the stimuli that control the level of flowering when the plants are young (0 DAT), may not work in the same way when they are more mature. It can be seen that young plants have opposing responses

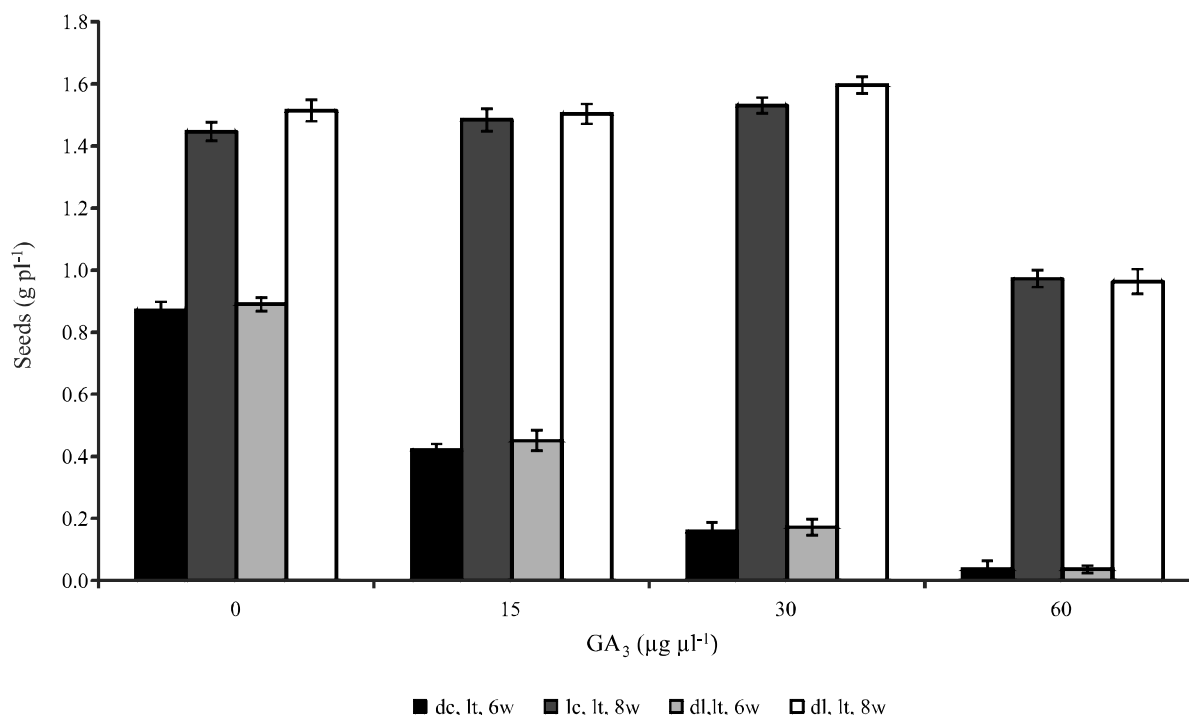
as regards the effects of the treatments on flowering and tassel elongation, while at 50 DAT, the stimuli have a similar effect on both.

Relationship between the different treatments and seed production

According to that seen in this study, *N. caerulea* requires low temperatures (4 °C) as a necessity in order to be stimulated to produce seeds (Figure 1). Additionally, longer exposure to low temperature conditions (4 °C), resulted in an increase on average of 60% in seed production per plant. In contrast, applications of GA₃, besides not promoting a significant increase in seed production when applied at a concentration of 60 µg µL⁻¹, caused a reduction in seed production, even when the plants were subjected to longer periods of exposure to low temperatures (4 °C).

There is little literature available which links vernalization and/or the application of gibberellins with the level of seed production. Walenta *et al.* (2002) found that the species *Aegilops cylindrica* L. AEGCY and *Triticum aestivum* L. "Madsen" produced a larger

Figure 1 - Seed production (g) in plants of *N. caerulea* under different periods of daily exposure to light (8 (dc) and 12 (dl) hours), temperatures (4 (lt) and 21 (wlt) °C), weeks of exposure to the above-mentioned combinations of light and temperature (6 (6w) and 8 (8w) weeks) and to different concentrations of gibberalin (GA₃) applied to the apical growth zone. Purdue University, USA, 2009



* Different lowercase letters denote a significant difference among averages within the same concentration of GA₃, and different uppercase letters denote significant differences among averages within the same growing condition, both at a 5% level of significance, according to the Scott-Knott test. The Coefficient of Variation (C.V.) for flowering percentage - 11.22%

quantity of seeds when subjected to vernalization at 4 °C for 10 weeks. However, according to these researchers, the former of the two species is less dependent on vernalization for seed production, being considered a facultative winter annual, different from the latter which is dependent on vernalization for flowering, and is not considered a facultative plant. Although not belonging to the same family as the species cited by Walenta *et al.* (2002), *N. caerulescens* shows similar behaviour to *T. aestivum*, and can be considered a non-facultative winter biennial, since it needs a certain period at low temperature both to germinate and to flower.

CONCLUSIONS

1. Low temperatures (4 °C) are essential for the induction of flowering and the production of seeds in de *N. caerulescens*;
2. GA₃, together with a long day (12 hours of light), in more mature plants, can provide a slight increase in the degree of flowering, not being effective however, in the production of seeds in *N. caerulescens*;
3. GA₃ causes an increase in main tassel length in plants exposed to both short or long days, this effect only being observed in plants of *N. caerulescens* which were not under low temperatures (21 °C);
4. The exposure time of the plants to low temperatures (4 °C) is very important in enhancing seed production in *N. caerulescens*, with longer exposure times being suggested;
5. The application of GA₃ at high levels adversely affects seed production in *N. caerulescens* under short days (8 hours of light).

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