Physiological, phenometric and productive changes in soybean crop due to the use of kinetin[1]

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ABSTRACT

A fast growth in the use of substances that increase soybean yield through physiological or morphological changes, such as cytokinin, has taken place in recent years. This study aimed at assessing the effect of the application time of cytokinin as kinetin on soybean crops. The experiment was carried out under field conditions, using four treatments (control and kinetin at the vegetative stages V_4, V_6 and $V_4 + V_6$), with five replications, in completely randomized blocks. Physiological (nitrate reductase and peroxidase activity, H_2O_2 content and lipid peroxidation) and phenometric parameters (leaf and root dry mass), as well as yield, were evaluated. The application of kinetin at V_4, V_6 or $V_4 + V_6$ increased the activity of the nitrate reductase enzyme and reduced stress (evaluated via lipid peroxidation and hydrogen peroxide content), in addition to raising the dry matter content. All these characteristics resulted in increased yield. However, the most significant effects were caused by the application of kinetin at V_6, which increased yield in 20 %, in relation to the control.

KEYWORDS: *Glycine max* (L.) Merrill; cytokinin; nitrate reductase; peroxidase.

RESUMO

Alterações fisiológicas, fenométricas e produtiva na cultura da soja devido ao uso de cinetina

Nos últimos anos, houve rápido crescimento na utilização de substâncias que buscam aumentar a produtividade de soja, como as citocininas, seja por alterações fisiológicas ou morfológicas. Objetivou-se avaliar o efeito da época de aplicação de citocinina na forma de cinetina, na cultura da soja. O experimento foi realizado em condições de campo, utilizando-se quatro tratamentos (controle e cinetina nos estágios vegetativos V_4, V_6 e $V_4 + V_6$), com cinco repetições, em blocos inteiramente casualizados. Foram realizadas avaliações fisiológicas (atividade de nitrato redutase e peroxidase, teor de H_2O_2 e peroxidação de lipídios) e fenométricas (massa de matéria seca de raiz e folhas), além da produtividade. A aplicação de cinetina em V_4, V_6 ou $V_4 + V_6$ proporcionou incremento na atividade da enzima nitrato redutase, redução do estresse (avaliado por meio da peroxidação lipídica e teor de peróxido de hidrogênio) e aumento no teor de massa de matéria seca. Todas essas características repercutiram no aumento da produtividade. Contudo, os efeitos mais expressivos foram causados pela aplicação de cinetina em V_6, que proporcionou incremento de 20 % na produtividade, em relação ao controle.

PALAVRAS-CHAVE: *Glycine max* (L.) Merrill; citocinina; nitrato redutase; peroxidase.

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is the most widely grown oilseed in the world, with 80 % of its production concentrated in the United States, Brazil and Argentina (USDA 2013). In Brazil, the average yield increases around 30 kg ha^{-1} year^{-1} and is currently about 3,000 kg ha^{-1}, which is far from the plant's genetic potential of up to 20,391 kg ha^{-1} (339.9 bags ha^{-1}) (Navarro Júnior & Costa 2002).

The yield potential of the crop is ruled by the genetic characteristics of its seeds. However, conditions in the growing environment, such as temperature, water regime, photoperiod, soil chemical and physical properties, as well as competition with other plants, decrease its yield to the mean values currently seen. Some studies have recorded yields of 10,760 kg ha^{-1} (Haegele & Below 2013). This indicates the possibility of exploiting the genetic characteristics of the crop through physiological management.

Within this context, physiological stimulation through the use of hormones is one of the strategies adopted. This is because hormones act as signaling

molecules in plants and may cause physiological changes, such as modulating nitrogen assimilation and antioxidant metabolism, among others, which affect carbon metabolism (dry mass accumulation and reallocation to the beans) and potentially increase yield (Soares 2014).

Cytokinins have been used to achieve this goal. The functions of these hormones include everything from promoting apical meristem growth to cell cycle regulation and nutrient mobilization, controlling senescence and the formation of root nodules in nitrogen-fixing plants (Zwack & Rashotte 2013, Cortleven & Schmülling 2015). Moreover, recent studies indicate that this hormone may modulate antioxidant metabolic pathways, reducing the stress level of plants (O'Brien & Benková 2013, Alazem & Lin 2015, Zwack & Rashotte 2015). Taken together, these functions demonstrate that using the hormone may cause changes that lead to a higher yield in plants. In addition, the growth stage at which these growth regulators are applied is also an important point to be studied, since its effect may be modified as a function of the time they are applied and the crop variety used.

In this respect, the present study aimed at assessing the effect of the stage of kinetin application on the physiological and phenometric parameters and yield of soybean crops.

MATERIAL AND METHODS

The experiment was conducted in an experimental field of the Centro Universitário de Patos de Minas, in Patos de Minas, Minas Gerais State, Brazil (18º36'36"S, 46º29'11"W and 890 m of altitude), from November 2014 to May 2015, in a Dystrophic Red Latosol (Oxisol) (45 % of clay, 36 % of silt and 19 % of sand) (Embrapa 2006). According to the Köppen's classification, Patos de Minas has a humid subtropical climate (Cwa), with an average annual rainfall of around 1,400 mm, as well as average, maximum and minimum annual temperatures of 21.1 ºC, 27.8 ºC and 16.3 ºC, respectively (Souza et al. 2005).

Maize was the summer crop grown in the experimental area in the previous year, used for silage and forage (*Brachiaria decumbens*) in the winter. Desiccation was performed at 25 days before planting, using Roundup Transorb® (glyphosate 480 g a.i. L^{-1}) at a dose of 4 L ha^{-1}.

The NS-7114-RR soybean cultivar was used, with spacing of 0.45 m between rows and a population of 380,000 plants at harvest. The variety used belongs to the maturity group 7.1, with undetermined growth habit and 120-day cycle. Prior to planting, seeds were treated with inoculant [Atmo® - *Bradyrhizobium japonicum* - SEMIA 5079 and 5080 (5 x 10^9 CFU mL^{-1})] at a dose of 0.5 mL kg^{-1} of seeds, fungicide + insecticide [Standak Top® - fipronil (250 g L^{-1}) + methyl thiophanate (225 g L^{-1}) + pyraclostrobin (25 g L^{-1})] at 2 mL kg^{-1} of seeds and molybdenum + cobalt + amino acids + algae extract [Exion DA® - molybdenum (75.6 g L^{-1}) + cobalt (7.56 g L^{-1}) + amino acids (163.8 g L^{-1}) + algae extract (37.8 g L^{-1})]. Soil samples were also collected before planting, to determine their chemical characteristics, in line with the methodology proposed by Embrapa (2011). According to the soil analysis (Table 1), liming to correct soil acidity was not required. The fertilizer used was 450 kg ha^{-1} of the formulation 08:30:10 + 3.26 % of Ca + 4.25 % of S + 0.2 % of B + 0.2 % of Zn.

For weed management, Roundup Transorb® [glyphosate (480 g L^{-1})] was applied at 14 and 35 days after sowing (DAS), at a dose of 2 L ha^{-1}. Pests were controlled using Fastac Duo® [acetamiprid (100 g L^{-1}) + alpha-cypermethrin (200 g L^{-1})], at a dose of 250 mL c.p. ha^{-1} + Pirate® [chlorfenapyr

Table 1. Results of soil analysis (0-20 cm) in the experimental area.

pH	P-Mel	Rem-P	K$^+$	Ca^{2+}	Mg^{2+}	Al^{3+}	H + Al
(H$_2$O)		mg dm^{-3}			cmol$_c$ dm^{-3}		
5.60	10.23	4.94	85.70	2.94	0.56	0.02	1.30
BS		t		T	V	m	OM
	cmol$_c$ dm^{-3}				%		dag kg^{-1}
3.72		3.74		5.02	74.11	0.53	3.12

Extractors: K and P-assimilable by Mehlich-1; remaining P in 10 mmol L^{-1} of a CaCl$_2$ solution; exchangeable Ca^{2+}, Mg^{2+} and Al^{3+} content extracted by KCl; potential acidity by calcium acetate; and total organic matter by titration.

(240 g L^{-1})] at 500 mL c.p. ha^{-1}, at 84 days after sowing.

Diseases were controlled by applying Opera® [pyraclostrobin (133 g L^{-1}) + epoxiconazole (50 g L^{-1})] at 54 DAS and Orkestra® [fluxapyroxad (167 g L^{-1}) + pyraclostrobin (333 g L^{-1})] at 300 mL c.p. ha^{-1} at 70 and 84 DAS. During the insecticide and fungicide application, 500 mL c.p. ha^{-1} of Assist® was added to the spray solution.

Each experimental plot was made up of four 7-meter-long rows spaced 0.45 m apart, totaling 12.6 m^2. The study area in each plot consisted of the center rows, disregarding 0.5 m at either end. A randomized block design was adopted, consisting of four treatments (T$_1$ - control - no application); T$_2$ - kinetin at V$_4$; T$_3$ - kinetin at V$_6$; T$_4$ - kinetin at V$_4$ + V$_6$), with 5 replications for each treatment. The kinetin dose applied was 50 mg ha^{-1}, based on existing commercial products used for soybean cultivation. Applications were carried out at 27 and 34 DAS, corresponding to the stages V$_4$ (fourth node, 3 fully developed trifoliate leaves) and V$_6$ (sixth node, 5 fully developed trifoliate leaves) (Fehr & Caviness 1977), respectively.

Leaf application was performed using a CO$_2$ pressurized backpack sprayer, with a 2.25 m-long boom and four spray nozzles, at a constant pressure of 2 bars. A spray volume of 200 L ha^{-1} was used for all applications.

Biochemical analyses were carried out on leaves from five plants in each block. The samples were homogenized and the material was used to assess the nitrate reductase activity (Mulder et al. 1959) at 39 and 53 DAS, as well as hydrogen peroxide (Alexieva et al. 2001) and lipid peroxidation (Heath & Packer 1968) at 89 DAS. Antioxidant enzyme activity was also determined at 89 DAS, based on the extraction of plant material (Kar & Mishra 1976). The same material was used to determine the total soluble protein content (Bradford 1976) and peroxidase activity (Teisseire & Guy 2000).

At 40, 67 and 91 DAS, four plants were collected from each plot to determine root and leaf dry mass. A furrow was dug alongside the plants during the collection process to avoid root loss. The plant parts were separated after collection and stored individually in paper bags. The different parts were dried using a standard forced air oven at 65 ºC, until constant weight.

At the end of the crop cycle (117 DAS), plants were manually harvested from the two center rows of each plot, disregarding 0.5 m at either end. Next, the plants were threshed using a tractor-mounted thresher. The water content of the beans was determined and yield (production per unit area) calculated with water content corrected to 13 % (0.13 g g^{-1}). The beans were weighed using a digital balance accurate to 0.01 g.

Statistical analyses were performed with the Sisvar® software (Ferreira 2010). The normality of Anova residuals and homogeneity between variances were analyzed using the Shapiro-Wilk and Levene's tests, respectively, both at 1 %. Next, analysis of variance was conducted and means were compared via standard deviation of the mean and the Tukey test at 5 %.

RESULTS AND DISCUSSION

At 39 DAS, the highest nitrate reductase activity recorded was for the kinetin treatment at V$_4$ + V$_6$. In this case, the increases observed were 16 %, 14 % and 79 %, in relation to the control, kinetin at V$_4$ and kinetin at V$_6$, respectively (Table 2). At 53 DAS, results were higher for all treatments, in relation to the control, with nitrate reductase activity increases of 75 %, 28 % and 51 %, respectively for the application at V$_4$, V$_6$ and V$_4$ + V$_6$.

Nitrate reductase is considered an important sensor capable of measuring the physiological status of plants, since the enzyme depends directly on energy from photosynthesis (Marschner 2012). Thus, the treatments that produced the highest nitrate reductase activity also influenced the physiological

Table 2. Nitrate reductase activity at 39 and 53 days after sowing (DAS), in soybean plants submitted to kinetin application at different stages.

Treatment	Nitrate reductase activity	
	39 DAS	53 DAS
	—— µg [N-NO$_2$] g^{-1} [fresh mass] h^{-1} ——	
Control	0.68 b[1]	0.66 d
Kinetin (V$_4$)	0.69 b	1.17 a
Kinetin (V$_6$)	0.44 c	0.86 c
Kinetin (V$_4$ + V$_6$)	0.79 a	1.01 b
MSD[2]	0.09	0.04
CV (%)	7.16	4.12

[1] Means followed by the same letter in the rows do not differ according to the Tukey test (p < 0.05); [2] MSD = minimum significant difference.

potential of the plant. The highest nitrate reductase activity, if compared to the control, was observed for the kinetin application at $V_4 + V_6$, suggesting that consecutive applications of the hormone are necessary to maintain high levels of the enzyme.

Researches using other cytokinins also demonstrate the effect of this hormone on the nitrate reductase activity. *Arabdopsis thaliana* plants grown in a medium containing benzyladenine, or submitted to leaf application of the hormone, showed a 3 to 14-fold increase in the activity of this enzyme (Yu et al. 1998), what is attributed to the effect of benzyladenine on the *Nia1* gene expression, which encodes the expression of nitrate reductase.

Maintaining the nitrate reductase activity is essential for nitrogen assimilation through nitrate in the soil. This affects the yield of soybean plants, despite their ability to obtain N via biological fixation, given the positive correlation between yield and nitrogen assimilation in the growth stage R_2. This occurs because, according to Fabre & Planchon (2000), there is a sharp decline in the biological nitrogen fixation after R_2, accompanied by increased nitrate assimilation up to the stage R_6. Additionally, the authors found a correlation between biological fixation in R_2 and R_5 and protein content in the beans. Although this correlation has not been observed for nitrogen assimilation via soil nitrate, these results indicate that it is vital to maximize the nitrogen metabolism during the reproductive phase, in systems with high yield potential.

Cytokinins may also be important in maintaining the physiological activity of plants. Synthesis of this hormone is induced by stress, prompting an increase in the nitrate reductase activity. This characteristic was observed in rice plants exhibiting overexpression of the *ipt* gene (which encodes cytokinin synthesis). Under water stress,

these plants exhibited a 3-fold increase in the nitrate reductase activity, when compared to wild plants (Reguera et al. 2013).

In addition to the aforementioned benefits, the use of kinetin also affected the oxidative metabolism (peroxidase activity, hydrogen peroxide content and lipid peroxidation). The use of kinetin in the stages V_4 and V_6 resulted in a greater peroxidase activity. The average increase in these treatments, in relation to the others, was 43 % (Table 3). Wheat leaves, in a medium containing a different cytokinin (10^{-1} M of benzylaminopurine), exhibited an increase in catalase and ascorbate peroxidase and reduced the hydrogen peroxide content, resulting in a lower percentage of electrolyte loss via membrane (Zavaleta-Mancera et al. 2007).

The decline in superoxide radicals produces hydrogen peroxide, a moderately reactive oxygen species, which causes oxidative stress. One of the effects of hydrogen peroxide is enzyme inactivation via the oxidation of thiol groups. However, at low concentrations, hydrogen peroxide acts as a signaling molecule for different plant processes, such as senescence, photorespiration, photosynthesis, stomatal movement, cell cycle, development and tolerance to different biotic and abiotic stresses. All stages of kinetin application reduced the hydrogen peroxide concentration, with declines of 20 %, 24 % and 34 %, respectively at V_4, V_6 and $V_4 + V_6$ (Table 3). As such, the rise in the peroxidase activity on kinetin application at V_4 or V_6 reduced hydrogen peroxide levels in these treatments. However, hydrogen peroxide levels were low in the treatment $V_4 + V_6$, where no changes in peroxidase activity were observed. This means that alternative pathways, such as catalase, may have acted to reduce the levels of this free radical, leading to a decrease in stress.

Table 3. Peroxidase activity, hydrogen peroxide content (H_2O_2) and lipid peroxidation at 89 days after sowing in soybean, after kinetin application at different growth stages.

Treatment	Peroxidase	Hydrogen peroxide	Lipid peroxidation
	μmol [purpurogallin] min^{-1} mg^{-1} [protein]	μmol g^{-1} [fresh mass]	nmol [TBARS] g^{-1} [fresh mass]
Control	0.114 b[1]	87.1 a	108.1 ab
Kinetin (V_4)	0.163 a	69.4 b	104.2 ab
Kinetin (V_6)	0.180 a	66.1 c	101.2 b
Kinetin ($V_4 + V_6$)	0.125 b	57.8 d	111.8 a
MSD[2]	0.03	3.17	7.56
CV (%)	9.82	2.41	3.79

[1] Means followed by the same letter in the rows do not differ according to the Tukey test (p < 0.05); [2] MSD = minimum significant difference.

Plant stress can be measured through lipid peroxidation, meaning that the higher the lipid peroxidation the greater the stress. Thus, the kinetin applications performed in the present study altered this variable, with the highest lipid peroxidation observed in the treatment $V_4 + V_6$, although there was no difference between this treatment and the control or V_4 (Table 3).

An increase in the cytokinin supply can stimulate the salicylic acid synthesis, as observed by Masuta et al. (1995) in tobacco plants. Salicylic acid promotes the accumulation of reactive oxygen species, which act as signaling molecules for the expression of genes related to hormone functions (Mittler 2002). The application of high concentrations of salicylic acid (more than 1 mM) results in high reactive oxygen species levels, causing a decline in the detoxification capacity, cell death and greater oxidative stress (Mittler 2002).

Analysis at 40 DAS showed that kinetin increased the root dry mass for all application stages, in relation to the control (Table 4).

At 67 DAS, the kinetin application at V_6 increased the root dry mass by 24 %, 23 % and 69 %, in relation to V_4, control and $V_4 + V_6$, respectively. An increase in root dry mass was observed for the kinetin treatment at $V_4 + V_6$, at 91 DAS, although there was no difference in relation to V_6 (Table 4).

The rise in the root dry mass accumulation after kinetin application may be related to a greater nitrate reductase activity and lower stress levels, which may have resulted in higher net photosynthesis in the treated plants. High root growth is important because it improves the plant ability to absorb water and nutrients. Plants with a substantial root surface area are more tolerant to possible stresses due to their superior soil exploitation ability (Larcher 2004), ensuring a better growth in roots. Additionally, large root systems may provide a greater nodule formation, leading to increased nitrogen assimilation (Fagan et al. 2015). As such, an increased accumulation of root dry mass, associated with a rise in the nitrate reductase activity and reduced stress levels, resulted in a greater leaf dry mass accumulation (Table 5).

At 40 DAS, all the treatments increased the leaf dry mass accumulation, in relation to the control. However, no difference was observed among V_4, V_6 and $V_4 + V_6$ (Table 5).

Kinetin application at 67 DAS showed the highest dry mass accumulation, in relation to the other treatments, and 18.8 %, in relation to the control (Table 5).

Cytokinins maintain membrane integrity, preventing the transport of proteases from the vacuole to the cytoplasm and hydrolyzing soluble proteins from plasma membranes, chloroplasts and mitochondria. Additionally, the hormone blocks fatty acid (phospholipids) oxidation, prevents membrane oxidation and degradation, inhibits the formation and breakdown of free radicals, such as superoxides (O_2^-) and hydroxyls (OH^-), inhibits chlorophyll degradation and promotes protein and nitrate reductase activity synthesis (Zavaleta-Mancera et al. 2007).

Moreover, leaf maintenance may be related to increased acid invertase activity. Cytokinins stimulate the activity of this enzyme, which catalyzes the cleavage of sucrose into hexoses, when it is released into sink organs (Lara et al. 2004). Thus, the application of cytokinins may keep leaves green for

Table 4. Root dry mass at 40, 67 and 91 days after sowing (DAS), in soybean plants submitted to kinetin application at different growth stages.

Treatment	Root dry mass		
	40 DAS	67 DAS	91 DAS
	g plant[-1]		
Control	0.49 b[1]	1.54 b	0.93 c
Kinetin (V_4)	0.75 a	1.54 b	0.99 bc
Kinetin (V_6)	0.72 a	1.91 a	1.08 ab
Kinetin ($V_4 + V_6$)	0.74 a	1.13 c	1.14 a
MSD[2]	0.10	0.08	0.11
CV (%)	8.12	5.77	7.73

[1] Means followed by the same letter in the rows do not differ according to the Tukey test (p < 0.05); [2] MSD = minimum significant difference.

Table 5. Leaf dry mass at 40, 67 and 91 days after sowing (DAS), in soybean plants submitted to kinetin application at different stages.

Treatment	Leaf dry mass		
	40 DAS	67 DAS	91 DAS
	g plant[-1]		
Control	2.11 b[1]	5.15 b	3.03 a
Kinetin (V_4)	2.30 a	4.59 b	3.16 a
Kinetin (V_6)	2.37 a	6.13 a	3.96 a
Kinetin ($V_4 + V_6$)	2.39 a	4.62 b	3.12 a
MSD[2]	0.12	0.96	0.36
CV (%)	5.75	10.0	6.20

[1] Means followed by the same letter in the rows do not differ according to the Tukey test (p < 0.05); [2] MSD = minimum significant difference.

longer and stimulate the release of energy into sink organs, what could lead to a greater yield.

According to Evans (1989), the production of dry matter by crops, in general, is primarily linked to nitrogen availability, in conjunction with greater CO_2 assimilation via photosynthesis. In soybeans, increased dry mass and leaf area during seed filling are reported by Nelson (1986) as a major factor for improving crop yield. All the kinetin treatments were superior to the control by an average of 16.9 % (Table 6). However, considering all the results together, the high dry mass accumulation and low stress level provided by the kinetin at V_6 makes this the most promising application stage.

The accumulation of dry matter from the stage R_1 and during R_5 is an important component in estimating soybean yield, primarily due to a greater light interception and photoassimilate partitioning to the reproductive organs, which contribute to optimizing yield (Board & Modali 2005).

The rise in the nitrate reductase activity (greater nitrogen assimilation), associated with a larger leaf area, may have increased the photosynthetic area, raising the net photosynthesis and resulting in a greater production of sugars for subsequent transport to the developing sink organs, in this case seed filling.

Figure 1 shows a mode of action for kinetin, whose application increased the nitrogen assimilation (Table 2). In addition, the hormone maintained the stress levels of the plants under study, being evident in the high peroxidase activity (Table 3) and low hydrogen peroxide content (Table 3), with a slight reduction in lipid peroxidation (Table 3). High nitrogen assimilation, associated with low stress levels, resulted in a greater root (Table 4) and leaf dry mass accumulation (Table 5), improving plant yield (Table 6).
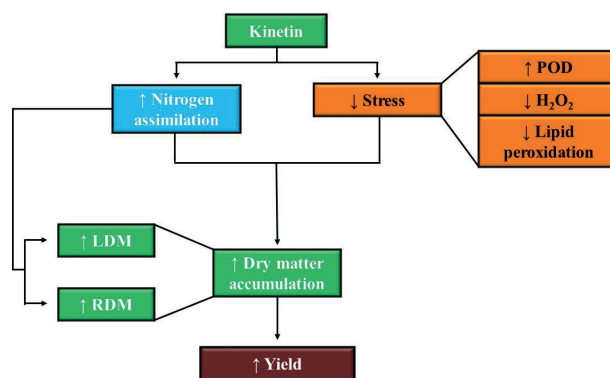

Figure 1. Mode of action of kinetin applied at different stages of soybean growth. POD: peroxidase; H_2O_2: hydrogen peroxide; RDM: root dry mass; LDM: leaf dry mass.

CONCLUSION

The kinetin application to soybean crops at V_4, V_6 or $V_4 + V_6$ increases the nitrate reductase activity, reduces stress levels and increases the dry mass accumulation, resulting in a high yield. However, the best effect on yield was observed for the kinetin application at V_6, with a 20 % increase, in relation to the control.

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Table 6. Soybean yield after kinetin application at different growth stages.

Treatment	Yield
	kg ha^{-1}
Control	3,540.6 b[1]
Kinetin (V_4)	4,124.3 a
Kinetin (V_6)	4,247.4 a
Kinetin ($V_4 + V_6$)	4,042.1 a
MSD[2]	217.7
CV (%)	5.91

[1] Means followed by the same letter do not differ according to the Tukey test ($p < 0.05$); [2] MSD = minimum significant difference.

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