

## Effects of photoperiod, potassium, and growth regulators on micropropagation of *Ocimum basilicum* L. 'Genovese'

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### Abstract

Medicinal plants are important in the pharmaceutical, cosmetic, and food industry owing to the diversity of their chemical composition. Basil (*Ocimum basilicum* L.) is a medicinal plant that is frequently used to produce cosmetics and food. However, the effects of factors such as photoperiod, growth regulators, and nutrients on growth during micropropagation are not comprehensively understood. The objective of this study was to assess the effect of different photoperiods and growth regulators on *in-vitro* growth and biochemical activity of the *O. Basilicum* 'Genovese' cultivated using a potassium-supplemented culture medium. The experiments were carried out in a completely causalized design and were conducted using a Murashige and Skoog culture medium plus sucrose and agar. In the first experiment, seeds were inoculated at three different photoperiods in the presence or absence of growth regulators. In the second experiment, growth media containing five different concentrations of potassium iodide (KI) (0, 25, 50, 75, and 100  $\mu$ M) were used. Basil seedlings grown at photoperiods of 16h and 24h produced more leaves and larger length and shoot mass, and addition of growth regulators showed no effect. However, the root systems produced the highest average values at a photoperiod of 16 h and when growth regulators were supplemented (0.23 g  $\pm$ 0,08). Antioxidant activity and the concentration of ascorbate peroxidase were higher under the 16 h light treatment and with addition of growth regulators. Addition of potassium to the growth medium had no beneficial effects on seedlings. Antioxidant activity was not affected by potassium concentrations, but it remained high (above 13.5%) when compared with the previous treatment. Catalase activity was the highest in seedlings grown at the highest concentrations of KI. The catalase enzyme is considered a stress indicator, so in higher concentrations of KI there is evidence that the basil plants may have had oxidative damage.

**Keywords:** antioxidants, basil, Catalase, Lamiaceae, luminosity.

**Abbreviations:** % SRL\_ free radical sequestration; APX\_ Ascorbate peroxidase; BAP\_ 6-benzylaminopurine; BOD\_ biochemical oxygen demand; Ca\_ calcium; CAT\_ Catalase; CL\_ chlorophyll; DMS\_ dry mass shoots; DPPH\_ diphenyl-1-picrylhydrazyl; DRM\_ dry root mass; FMS\_ fresh mass shoots; FRM\_ fresh root mass; K\_ potassium; KI\_ potassium iodide; LS\_ length of shoots; Mg\_ magnesium; MS\_ medium Murashige and Skoog; N\_ nitrogen; NAA\_  $\alpha$ -naphthaleneacetic acid; NBT\_ nitroblue tetrazolium; NL\_ Number of leaves; NS\_ Number of shoots; P\_ phosphorus; SISVAR 5.6\_ computer statistical analysis system; SOD\_ Superoxide dismutase.

### Introduction

Plant tissue culture is a biotechnological technique that facilitates growing of plants in culture media under controlled conditions to produce seedlings in a short amount of time and at high propagation rates (George et al., 2008). Furthermore, this method allows the production of pathogen-free plants of high and standardized quality (Cassells, 2012). Medicinal plants have pharmaceutical properties and have been consumed as natural remedies over millennia (Makri and Kintzios, 2008). More recently, with the advance of chemistry, biochemistry, and techniques for determining chemical compounds, medicinal plants have been used more frequently by the pharmaceutical, cosmetic, and food industry (Alvarez, 2014).

*Ocimum basilicum* is a medicinal plant of the family Lamiaceae and has antioxidant, antimicrobial, and antitumor properties (Suppakul et al., 2003; Hussain et al., 2008; Mahmoud, 2013; Tenore et al., 2017) owing to the presence of a variety of chemical compounds such as phenylpropanoids, terpenoids, and rosmarinic acid (Trettel et al., 2017; Pagano et al., 2018). The genus *Ocimum* comprises about 50-60 species with different chemical and morphological characteristics (Makri and Kintzios, 2008). One example is the cultivar 'Genovese', which has large leaves with a leaf area of more than 300 cm<sup>2</sup> in a mature plant, and therefore a considerable amount of biomass accumulates in the leaves (Furlan et al., 2013).

Advances in cultivation techniques and propagation of medicinal plants have not occurred at the same speed as in the industrial sector, and information is lacking regarding factors such as cultivar choice, pest control, diseases, and plant spacing (Makri and Kintzios, 2008; Ncube et al., 2012). Particularly regarding micropropagation, very few protocols have been developed, and the existing studies are inconclusive. This problem also concerns micropropagation of members of the family Lamiaceae, particularly of the genus *Ocimum*. When propagation protocols are established, typically growth medium components such as macro- and micronutrients, growth regulators, sugars, and others are examined. Plant organogenesis after propagation, however, depends on three crucial factors: genetics, culture medium components, and physical growth medium (George et al., 2008).

Growth medium composition affects plant physiology and morphology, particularly if the used compounds in their respective concentrations cause stress, which may result in an increase or decrease of secondary compounds and oxidative damage (Gill and Tuteja et al. 2010), which may severely damage the explants (Matkowski, 2008). Cytokinins and auxins are the most frequently used growth regulators for tissue culture, and they can influence both plant morphology and growth responses such as shoot and root emission (Motte et al., 2014). Regarding macronutrients, potassium is particularly important for the functioning of various enzymes, maintaining the homeostatic equilibrium, and opening and closing of the stomata (Amtmann and Armengaud, 2009).

The factor 'growth conditions' typically comprises the physical factors that seedlings are exposed to, including temperature, humidity, photoperiod, spectral light range, and light intensity (George et al., 2008). The term photoperiod indicates the amount of time per day during which plants are exposed to light (Adams and Langton, 2005). In an *in-vitro* environment, effective photosynthesis can be reduced, however, light is essential and plant cells contain proteins termed phytochromes which respond to light and thereby trigger physiological and biochemical responses (Adams and Langton, 2005).

Growth chamber light is typically produced by LED lamps, which causes substantial energetic costs for laboratories and companies that produce seedlings through micropropagation; thus, optimization of the photoperiod may help reduce the costs of micropropagation (Adams and Langton, 2005; George et al., 2008). Furthermore, it is not comprehensively understood how the photoperiod affects the biochemical activity of medicinal plants, particularly regarding antioxidant enzymes including free radical scavengers such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) (Gill and Tuteja 2010) and antioxidant compounds (Matkowski, 2008). These aspects have been studied in some depth under conventional cultivation regimes (Adams and Langton, 2005), however, studies under *in-vitro* conditions are scarce. Photoperiod and addition of potassium and growth regulators likely affect *O. basilicum* growth, which may help produce plants of high physiological quality (Jo et al., 2008). Moreover, these factors may be expected to affect the function of antioxidant mechanisms in *O. basilicum* plants, and we predicted that the activity of enzymes and chemical compounds should increase in treatments that cause more damage to the seedlings. The objective of this study was to

evaluate *in-vitro* growth and biochemical activity in *O. basilicum* 'Genovese' supplemented with potassium at different photoperiods and with different amounts of growth regulators.

## Results and Discussion

### Photoperiod and growth regulators

The interaction of culture medium and photoperiod affected the assessed characteristics. The amount of produced leaves increased by approximately 50% in plants grown in media without growth regulators and in seedlings grown under 16 h of light (T3; Table 2). Seedling length and dry and fresh mass increased in plants grown without regulators and with 24 h and 16 h light (T3 and T1, respectively; Table 2). However, for the growth of the root system, addition of growth regulators was beneficial mainly in T5 and T6 (Table 2). Callus formation which is common in plants of the Lamiaceae family was observed in all treatments with growth regulators, and the highest average values were observed in treatments T5 and T6 (Table 2). The number of shoots and the chlorophyll index were not influenced by any of the assessed factors.

We observed a substantial increase in the production of new leaves in *O. basilicum* 'Genovese' seedlings grown at 16h of light. The prediction that addition of growth regulators should increase this effect was not confirmed. The amount of leaves is particularly important in *in-vitro* production of basil because leaves contain essential oil-producing glands, which is of interest to the industry (Zuzarte et al., 2010; Amaral-Baroli et al., 2016). Numerous studies have been conducted on the effects of photoperiod on the flowering process; however, the effect of photoperiod on plant growth has received little attention (Adams and Langton, 2005). In the few existing studies the rate of leaf emission as well as the leaf area tended to increase in periods of 16/8 or 12/12 (Vaillant et al., 2005; Jo et al., 2008). According to Adams and Langton (2005), the increase in the photoperiod should promote an increase in meristematic activity at the apex of shoots, which would promote leaf growth. However, its continuous activity may lead to photo-oxidation, chlorosis, and foliar necrosis (Velez-Ramirez et al., 2011).

With 24h light, *O. basilicum* 'Genovese' leaves increased in length, suggesting increased production of gibberellins. However, in *Chrysanthemum morifolium* Ramat. 'Ellen', the same treatment led to a reduction in branch length (Kurilčik et al., 2008). Increased shoot biomass production in treatments T1 and T3 may be due to a higher frequency of cell divisions caused by an optimum of endogenous hormone concentrations (Motte et al., 2014). Exogenous supplementation with growth regulators substantially reduced the biomass increment, which was more severe in plants grown at longer photoperiods. If not metabolized by the plant, growth regulators exert adverse and toxic effects (Motte et al., 2014). Correspondingly, the dry weight of *C. morifolium* Ramat. 'Ellen' increased more than twice at photoperiods of 16h (Kurilčik et al., 2008).

In the root system of basil plants, 8 h of light and growth regulator supplementation increased root mass, which was likely due to the beneficial effects of auxins that elicit formation of lateral roots (Fukaki and Tasaka, 2009; Motte et al., 2014). Similar effects of the photoperiod on auxins were observed in *Plumbago zeylanica* L. (Saxena et al., 2000).

The amount of absorbed N differed substantially between shoots and roots; on average, shoots contained 30% more N, regardless of treatment (Table 3). Treatments T4 ( $64.40 \text{ g.Kg}^{-1} \pm 0.21$ ) and T6 ( $58.06 \text{ g.Kg}^{-1} \pm 5.23$ ) showed the highest N uptake rates in shoots, and treatments T2 ( $45.90 \text{ g.Kg}^{-1} \pm 0.49$ ) and T4 ( $41.34 \text{ g.Kg}^{-1} \pm 7.73$ ) showed the highest amount of N in roots (Table 3). P was higher in treatments T3 ( $3.17 \text{ g.Kg}^{-1} \pm 0.57$ ) and T4 ( $2.63 \text{ g.Kg}^{-1} \pm 0.09$ ) than in the other treatments, in both shoots and roots (Table 3).

Antioxidant activity was substantially increased in treatment T6 ( $17.29 \pm 4.48$ ), whereas no significant difference was observed among the other treatments (Table 4). Antioxidant activity in *O. basilicum* 'Genovese' may be related to two factors: 1) stress caused by the treatments, or 2) an elicitation factor (Patel and Krishnamurthy, 2013; Alvarez, 2014). If culture medium and photoperiod cause stress, one of the defense mechanisms of plants is to increase the production of secondary metabolites which can oxidize free radicals that otherwise damage lipids, proteins, and nucleic acids (Noctor et al., 2018). An increase in antioxidant activity by more than 60% (T6) compared to T3 indicated increased production of secondary metabolites to mitigate damage caused by free radicals.

Plants of the genus *Ocimum*, and particularly basil, are known to produce terpenoids such as linalool (Trettel et al., 2017) and phenylpropanoids such as eugenol and methyl eugenol (Trettel et al., 2017; Trettel et al., 2018b), and they can also produce several phenolic acids such as rosmarinic acid (Kiferle et al., 2013; Pagano et al., 2018). Antioxidant capacity of all of these compounds has been shown previously (Javanmardi et al., 2003; Tenore et al., 2017); therefore, it is possible that these compounds mitigated the respective effects in T6, which would explain the higher activity observed in this treatment.

The functions of antioxidant enzymes have been studied in-depth in plants under natural conditions (Demidchik, 2015; Noctor et al., 2018); however, less is known about their functions in *in-vitro* environments and under different photoperiods. Understanding these mechanisms may help increase plant productivity. The results of these assays showed variable responses depending on the treatment. APX concentration was higher in T6 (1.5 mM), whereas enzyme concentration was similar among the other treatments and was below 0.2 mM (Fig. 1A); CAT concentration was highest in the T2 and T5 treatments (above 0.3 mM; Fig.1B). SOD activity was highest activity, except in T6, the average of the other treatments was above 250U (Fig.1C).

In line with antioxidant activity, APX activity increased eight-fold in treatment T6, compared with the other treatments. This enzyme is part of the second phase of free radical scavenging and catalyzes  $\text{H}_2\text{O}_2$  to water using ascorbate as an electron donor (Demidchik, 2015; Noctor et al., 2018). Interestingly, APX did not exceed 0.35 mM (Fig.1.B) in any other treatment, and APX activity was 4.5-times higher than those of CAT. CAT and APX concentration were approximately 0.2 mM, each, in all other treatments.

The functions of antioxidant enzymes have been studied in-depth previously (Demidchik, 2015; Noctor et al., 2018); however, optimal timing and enzymatic succession in plants remains unknown and is very difficult to examine because processes in plant cells are dynamic and occur synchronously. Plants have several antioxidant mechanisms to promote free radical scavenging (Ahangar and Agarwal,

2017). Determining which pathway is the most important one in any given situation is not straightforward because this may depend on other metabolic processes that are ongoing at that moment (Aghaei et al., 2009). As an example of this phenomenon, APX concentration in *Melissa officinalis* L. plants subjected to different NaCl concentrations (0-200 mM) was not affected by the treatment, whereas CAT increased to up to 150 mM after a near-50% decrease (Bonacina et al., 2017), and SOD increased in seedlings treated with 150 and 200 mM NaCl. In this case, the most important enzyme appeared to be CAT followed by SOD, as both enzymes seemed to neutralize free radicals, along with other mechanisms. Taken together, antioxidant enzyme kinetics also depend on other metabolic processes.

#### **Supplementation with KI in culture medium**

Potassium is an important macronutrient as it is a co-factor of several enzymes; it is required in the processes of plant cell osmoregulation (Amtmann and Armengaud, 2009), and culture media typically contain a defined amount of K. We predicted that K supplementation would increase seedling growth, however, excessive amounts of K may also elicit adverse responses. So far, the concentration of K in growth media of medicinal plants has received little attention (Silva et al., 2017).

Addition of K to the culture medium showed no beneficial effect on growth and did not affect the assessed morphological traits. Leaf number and shoot dry mass decreased with increasing K concentration (Table 5). Length and fresh mass of the shoots, fresh and dry mass of the roots, and the chlorophyll index did not differ significantly between treatments (Table 5). Interestingly, no callus formation was observed in any of the treatments. Similar results were observed after *in-vitro* cultivation of *O. basilicum* 'Red Rubin' (Silva et al., 2017) and *Curcuma longa* L. (unpublished data) using similar KI concentrations as in the present study. Seedlings of *Cattleya loddigesii* Lindley grown at different concentrations of K silicate showed an increase in the number of leaves, growth, and shoot mass at concentration of up to  $1 \text{ mg.L}^{-1}$  (Alves et al., 2016). According to Amtmann and Armengaud (2009) and Malvi (2011), excess K affects biosynthesis and metabolic allocation.

Elemental nutrients are absorbed at different velocities, and the presence of one nutrient may inhibit or enhance the rate of absorption of a different nutrient. Inhibition can occur when entry into the root cells takes place via the same site of absorption, resulting in absorption competition or antagonism. Synergistic enhancement typically occurs between nutrients that are absorbed at different sites (Malvi, 2011; Rietra et al., 2017). Regardless of the treatment, the N was absorbed at greater quantities than K, with 69.25% absorbed by the shoots and 73.53% by the roots. Nitrogen uptake showed similar average values in all treatments, apart from treatment T1 that did not receive additional K and showed higher N uptake ( $48.74 \text{ g.Kg}^{-1} \pm 4.18$ ). In roots, we observed a trend of N uptake to increase with increasing concentrations, with a maximum absorption of ( $47.95 \text{ g.Kg}^{-1} \pm 1.10$ ) at  $100 \mu\text{M}$  (Table 6).

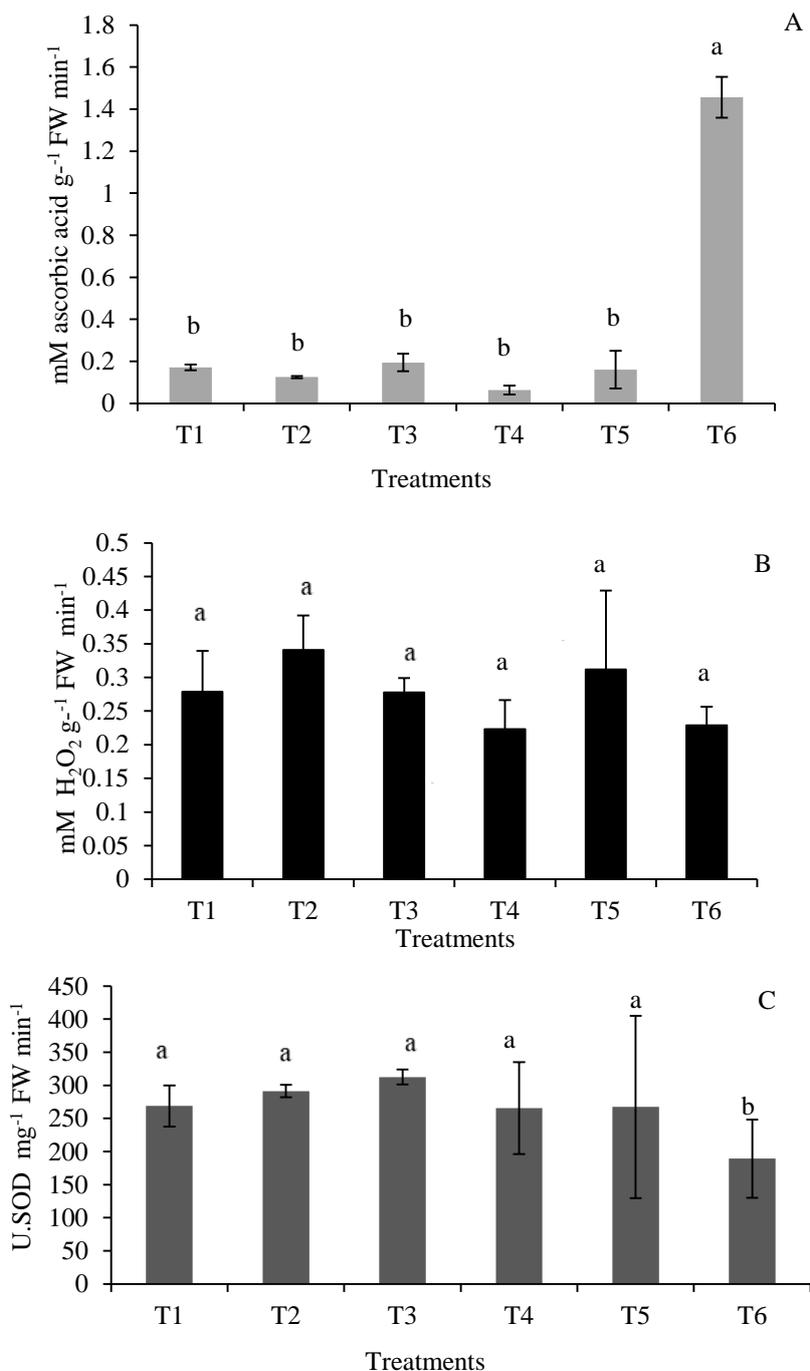
Antagonism typically occurs between Ca or Mg uptake and K uptake, as these minerals compete for the same site of absorption (Malvi, 2011); therefore, we predicted a respective effect in seedlings of *O. basilicum* 'Genovese'. Shoot Ca content, however, was similar among all

treatments, and root Ca content increased slightly at higher K concentrations (Table 6). Shoot Mg content also did not differ significantly between the treatments, and root Mg content increased at higher K concentrations (Table 6).

Additional K thus did not negatively affect absorption of Ca or Mg in *O. basilicum* 'Genovese'. Ca deficiency typically manifests as death of the apical egg yolk, chlorosis, and internodal

**Table 1.** Culture medium and photoperiods for growth and biochemical activity *O. basilicum* 'genovese'.

	Treatments 1	Treatments 2	Treatments 3	Treatments 4	Treatments 5	Treatments 6
MS (%)	100	100	100	100	100	100
BAP (mg.L <sup>-1</sup> )	0.0	0.0	0.0	0.4	0.4	0.4
NAA (mg.L <sup>-1</sup> )	0.0	0.0	0.0	0.2	0.2	0.2
Photoperiod	24 horas light	12h light /12h dark	16h light /8h dark	24 horas light	12h light /12h dark	16h light /8h dark



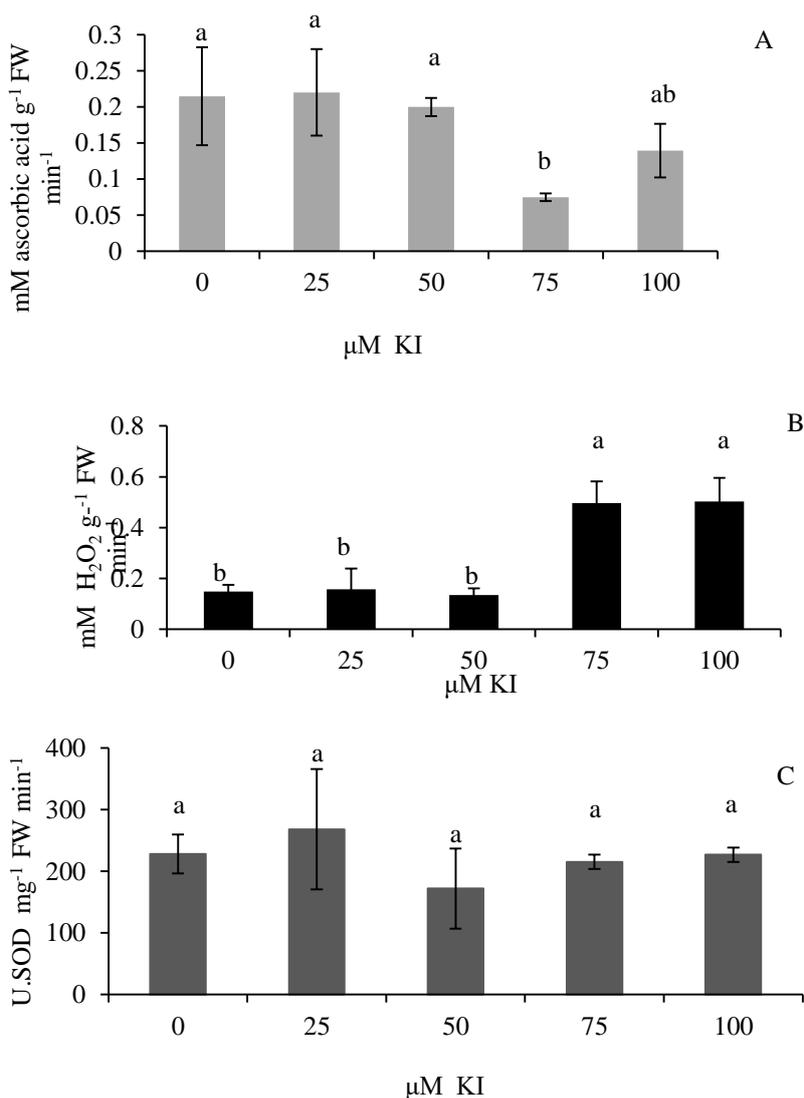
**Fig 1.** Antioxidant enzymes activity APX (A), CAT (B) and SOD (C) obtained from fresh leaves of *O. basilicum* 'genovese' as a function of different formulations of MS medium and photoperiods. Means followed by the same letter in the column do not differ by Tukey's test 5%. **T1** -24 h light; **T2** - 12h light + 12h dark; **T3** - 16 h light + 8 h dark - (T1: T3 MS 100%, sucrose 30.0 g.L<sup>-1</sup>, agar 6.5 g.L<sup>-1</sup>); **T4** -24 h light; **T5** - 12h light + 12 h dark; **T6** - 16 h light + 8 h dark - (T4:T6 MS 100%, sucrose 30.0 g.L<sup>-1</sup>, agar 6.5 g.L<sup>-1</sup>; BAP 0.4 g.L<sup>-1</sup> and NAA 0.2 g.L<sup>-1</sup>)

**Table 2.** Number of leaves (NL), shoots (NS), length of shoots (LS), fresh mass shoots (FMS), dry mass shoots (DMS), fresh root mass (FRM), dry root mass (DRM), callus fresh (CF) and callus dry (CDM) and chlorophyll (CL) of *O. basilicum* 'genovese' as a function of different formulations of MS medium and photoperiods.

Treatments	NL	NS	LS (mm)	FMS (g)	DMS (g)
T1	16.46 ± 3.54 <sup>b*</sup>	1.00 ± 0.00 <sup>a</sup>	61.48 ± 5.66 <sup>a</sup>	4.03 ± 0.68 <sup>ab</sup>	0.31 ± 0.03 <sup>a</sup>
T2	13.27 ± 2.42 <sup>b</sup>	0.75 ± 1.50 <sup>a</sup>	42.54 ± 5.88 <sup>b</sup>	3.12 ± 0.33 <sup>bc</sup>	0.23 ± 0.04 <sup>b</sup>
T3	24.33 ± 2.19 <sup>a</sup>	0.00 ± 0.00 <sup>a</sup>	57.52 ± 1.29 <sup>ab</sup>	4.40 ± 0.61 <sup>a</sup>	0.36 ± 0.04 <sup>a</sup>
T4	12.87 ± 3.45 <sup>b</sup>	1.50 ± 1.73 <sup>a</sup>	53.65 ± 5.70 <sup>ab</sup>	1.15 ± 0.61 <sup>d</sup>	0.07 ± 0.03 <sup>c</sup>
T5	13.13 ± 3.32 <sup>b</sup>	1.00 ± 1.15 <sup>a</sup>	42.18 ± 7.01 <sup>b</sup>	2.21 ± 0.45 <sup>bc</sup>	0.12 ± 0.03 <sup>c</sup>
T6	12.75 ± 4.17 <sup>b</sup>	0.25 ± 0.50 <sup>a</sup>	59.11 ± 13.86 <sup>ab</sup>	4.67 ± 0.56 <sup>a</sup>	0.20 ± 0.02 <sup>b</sup>
Treatments	FRM (g)	DRM (g)	CF (g)	CDM (g)	CL
T1	3.00 ± 1.05 <sup>ab</sup>	0.13 ± 0.04 <sup>ab</sup>	0.00 ± 0.00 <sup>b</sup>	0.00 ± 0.00 <sup>c</sup>	20.43 ± 2.39 <sup>a</sup>
T2	1.86 ± 0.79 <sup>b</sup>	0.08 ± 0.03 <sup>b</sup>	0.00 ± 0.00 <sup>b</sup>	0.00 ± 0.00 <sup>c</sup>	21.02 ± 1.40 <sup>a</sup>
T3	4.30 ± 0.50 <sup>a</sup>	0.13 ± 0.04 <sup>ab</sup>	0.00 ± 0.00 <sup>b</sup>	0.00 ± 0.00 <sup>c</sup>	26.71 ± 2.38 <sup>a</sup>
T4	3.02 ± 0.44 <sup>ab</sup>	0.12 ± 0.03 <sup>b</sup>	1.43 ± 0.98 <sup>b</sup>	0.10 ± 0.13 <sup>bc</sup>	24.11 ± 1.68 <sup>a</sup>
T5	4.04 ± 0.97 <sup>a</sup>	0.15 ± 0.04 <sup>ab</sup>	3.95 ± 0.46 <sup>a</sup>	0.21 ± 0.02 <sup>ab</sup>	22.16 ± 3.25 <sup>a</sup>
T6	3.00 ± 0.60 <sup>ab</sup>	0.23 ± 0.08 <sup>a</sup>	5.47 ± 1.19 <sup>a</sup>	0.25 ± 0.09 <sup>a</sup>	21.88 ± 4.66 <sup>a</sup>

\* Means followed by the same letter in the column do not differ by Tukey's test 5%

**T1** -24 h light; **T2** - 12h light + 12h dark; **T3** - 16 h light + 8 h dark - (T1: T3 MS 100%, sucrose 30.0 g.L<sup>-1</sup>, agar 6.5 g.L<sup>-1</sup>); **T4** -24 h light; **T5** - 12h light + 12 h dark; **T6** - 16 h light + 8 h dark - (T4:T6 MS 100%, sucrose 30.0 g.L<sup>-1</sup>, agar 6.5 g.L<sup>-1</sup>; BAP 0.4 g.L<sup>-1</sup> and NAA 0.2 g.L<sup>-1</sup>).



**Fig 2.** Antioxidant enzymes activity APX (A), CAT (B) and SOD (C) obtained from fresh leaves *O. basilicum* 'genovese' as a function of different potassium iodide (KI) concentrations. Means followed by the same letter in the column do not differ by Tukey's test 5%.

necrosis in new leaves (White and Broadley 2003), and Mg deficiency elicits chlorosis between veins in older leaves with the nerves showing deep green coloration (Guo et al., 2016); however, no signs of Ca or Mg deficiency were observed. Any negative responses observed in the treatments were not due to Ca or Mg deficiencies, but caused by other metabolic and physiological processes due to excess K.

We previously reported (Experiment 1) the importance of determining antioxidant activity in an assay and how this can be used as an indicator of stress. In the present study, however, antioxidant activity was not affected by the treatments (Table 7). The control treatment produced a value of 14.43% which may be an increase since this medium contained a different growth regulator than the first assay (Alvarez, 2014), or a momentary factor of undetected stress in this treatment. We discussed above the difficulties in understanding the dynamics of free radical control by plant cells as a function of innumerable simultaneous processes that occur within each cell. In this experiment, the response to additional K caused was different than in the first experiment.

Regarding antioxidant enzymes, responses were affected by the treatments. APX concentrations were above 0.2 mM in the control treatment and in the two treatments with the lowest KI concentrations (25 and 50  $\mu\text{M}$ ). At KI concentrations of 75 and 100  $\mu\text{M}$ , APX decreased to values between 0.1 and 0.15 mM (Fig 2A). The response of CAT showed a reversed pattern with the highest KI concentrations of 75 and 100  $\mu\text{M}$  producing higher CAT values of above 0.5 mM, with similar values in the other treatments (about 0.15 mM; Fig. 2B). SOD activity was below 200 U only in plants cultured in the medium with 50  $\mu\text{M}$  KI, whereas KI concentrations of 25  $\mu\text{M}$  increased SOD activity to over 250 U (Fig.2C).

At lower KI concentrations, APX seemed to be more important as its concentrations doubled in the three lowest KI concentrations, in contrast to CAT which at KI concentrations of 75 and 100  $\mu\text{M}$  increased four-fold. The elimination of reactive oxygen species by antioxidant enzymes may improve tolerance to agents that cause stress. In seedlings of *Triticum aestivum* L. subjected to different K concentrations (0, 20, 40, and 60  $\text{kg}\cdot\text{ha}^{-1}$ ) APX, CAT, and SOD enzyme activity increased, facilitating optimal plant growth (Ahanger and Agarwal, 2017). We therefore suggest that the adverse effects of KI on *O. basilicum* 'Genovese' growth did not occur as potential radicals would have been neutralized by antioxidant enzymes.

## Materials and Methods

### Seed collection and processing

The experiments were conducted in the laboratories of Molecular Biology and Plant Tissue Culture of the Paranaense University. Seeds of the cultivar 'Genovese' were obtained from the commercial supplier (Horticeres<sup>®</sup> São Paulo, Brazil) and were subjected to an asepsis procedure using 2% sodium hypochlorite solution for 15 min

under stirring; the seeds were then washed four times using autoclaved water.

### Experiment 1: Growth regulators and photoperiod

Three different photoperiods and two formulations of Murashige and Skoog culture medium (Murashige and Skoog, 1962) were used (Table 1). All treatments were supplemented with 30  $\text{g}\cdot\text{L}^{-1}$  sucrose and 6.5  $\text{g}\cdot\text{L}^{-1}$  agar (Kasvi<sup>®</sup> Paraná, Brazil), and the pH was adjusted to 5.8. These treatments were designed based on a previous study (Trettel et al., 2018a).

After preparation of the medium, glass vials containing culture medium were autoclaved at 121 °C for 20 min and were then used for seed inoculation which was performed in an aseptic chamber. Four seeds were placed in one flask containing 50 mL of the culture medium. The flasks were then closed using clear plastic lids and were sealed using PVC film. The flasks were maintained for 80 days at the biochemical oxygen demand (BOD) at 25 °C and under three different photoperiods: 24 h, 12 h, or 16 h. The BOD was equipped with white fluorescent 30-W lamps 84.60  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$  (Philips<sup>®</sup> Amsterdam, Netherlands).

### Experiment 2: Potassium iodide concentrations

Seeds subjected to the asepsis treatment were placed in flasks containing Murashige and Skoog medium supplemented with 30  $\text{g}\cdot\text{L}^{-1}$  sucrose, 0.1  $\text{mg}\cdot\text{L}^{-1}$  6-Benzylaminopurine (BAP) (Sigma Aldrich<sup>®</sup> Hamburg, Germany), and 6.5  $\text{g}\cdot\text{L}^{-1}$  agar, and the pH was adjusted to 5.8. Five concentrations of potassium iodide (KI) were used: 0, 25, 50, 75, and 100  $\mu\text{M}$ . The cultures were maintained in a growth chamber at  $25 \pm 2$  °C for 80 days. The photoperiod was 24 h, and white fluorescent 20-W lamps (Empalux<sup>®</sup> Curitiba, Brazil) at a light intensity of 72.0  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ .

### Biometric and physiological characteristics

After 80 days, the following characteristics were assessed in each experiment: leaf number, shoot number, shoot length, fresh shoot mass, shoot dry mass, total dry mass, fresh mass, dry callus mass, and chlorophyll content. Root length was measured using a digital caliper, and root and shoot dry mass was recorded after drying to constant weight at 65 °C. The chlorophyll index was obtained using a chlorophyll meter (ClorofiLOG<sup>®</sup> model CFL1030; Falck<sup>®</sup> Porto Alegre, Brazil).

### Determination of macro - and micronutrients

Shoots and roots of each treatment were collected at the end of the experiment and were oven-dried to constant weight under air circulation at 65 °C. In plants of experiment 1, nitrogen (N), and phosphorus (P) were measured. In experiment 2 (N) nitrogen, potassium (K), calcium (Ca), and magnesium (Mg) were measured. The samples were sent to the Plant Tissue Laboratory of the University of São Paulo. Nutrient concentrations were measured according to a

previous study (Malavolta, 1980). All analyses were performed in duplicates.

#### Analysis of antioxidant activity using a DPPH test

This analysis was performed 80 days after the start of each experiment. Approximately 0.5 g of fresh leaves were macerated in liquid nitrogen to obtain the extract

(Magalhães et al., 2017). Antioxidant activity was assessed based on the extinction by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH 60 µM) as shown by Rufino et al. (2009). A 0.1-mL aliquot of the extract was used, which was transferred to a test tube in a dark environment, and 3.9 mL of the DPPH radical (0.06 mM) was added. Measurements of absorbance reduction

**Table 3.** Nitrogen (N) and Phosphorus (P) measured at the end of 80 days of shoots (S) and roots (R) of *O. basilicum* 'genovese' as a function of different formulations of MS medium and photoperiods.

Treatments	g.Kg <sup>-1</sup>			
	N		P	
	B	R	B	R
T1	54.63±0.04 <sup>abc*</sup>	39.87±2.14 <sup>ab</sup>	2.50±0.39 <sup>a</sup>	2.55±0.23 <sup>a</sup>
T2	50.94±4.39 <sup>bc</sup>	45.90±0.49 <sup>a</sup>	3.04±0.47 <sup>a</sup>	2.27±0.09 <sup>a</sup>
T3	51.39±0.41 <sup>bc</sup>	36.77±2.26 <sup>ab</sup>	2.60±0.06 <sup>a</sup>	1.99±0.17 <sup>ab</sup>
T4	64.40±0.21 <sup>a</sup>	41.34±7.73 <sup>a</sup>	2.92±0.11 <sup>a</sup>	2.63±0.09 <sup>a</sup>
T5	44.87±1.55 <sup>c</sup>	25.76±2.93 <sup>b</sup>	2.75±0.06 <sup>a</sup>	1.29±0.14 <sup>b</sup>
T6	58.06±5.23 <sup>ab</sup>	39.21±2.37 <sup>ab</sup>	3.17±0.57 <sup>a</sup>	1.60±0.21 <sup>bc</sup>

\*Means followed by the same letter in the column do not differ by Tukey's test 5%. **T1** -24 h light; **T2** - 12h light + 12h dark; **T3** - 16 h light + 8 h dark - (T1: T3 MS 100%, sucrose 30.0 g.L<sup>-1</sup>, agar 6.5 g.L<sup>-1</sup>); **T4** -24 h light; **T5** - 12h light + 12 h dark; **T6** - 16 h light + 8 h dark - (T4:T6 MS 100%, sucrose 30.0 g.L<sup>-1</sup>; agar 6.5 g.L<sup>-1</sup>; BAP 0.4 g.L<sup>-1</sup> and NAA 0.2 g.L<sup>-1</sup>).

**Table 4.** Total antioxidant activity from 2,2-difenil-1-picrilhidrazil (DPPH) obtained after 80 days of fresh leaves *O. basilicum* 'genovese' as a function of different formulations of MS medium and photoperiods.

Treatments	Total antioxidant activity (%)
T1	5.91±1.84 <sup>b*</sup>
T2	6.37±2.24 <sup>b</sup>
T3	4.41±1.32 <sup>b</sup>
T4	4.41±1.58 <sup>b</sup>
T5	1.94±1.44 <sup>b</sup>
T6	17.29±4.48 <sup>a</sup>

\*Means followed by the same letter in the column do not differ by Tukey's test 5%. **T1** -24 h light; **T2** - 12h light + 12h dark; **T3** - 16 h light + 8 h dark - (T1: T3 MS 100%, sucrose 30.0 g.L<sup>-1</sup>, agar 6.5 g.L<sup>-1</sup>); **T4** -24 h light; **T5** - 12h light + 12 h dark; **T6** - 16 h light + 8 h dark - (T4:T6 MS 100%, sucrose 30.0 g.L<sup>-1</sup>; agar 6.5 g.L<sup>-1</sup>; BAP 0.4 g.L<sup>-1</sup> and NAA 0.2 g.L<sup>-1</sup>).

**Table 5.** Number of leaves (NL), shoots (NS), length of shoots (LS), fresh mass shoots (FMS), dry mass shoots (DMS), fresh root mass (FRM) dry root mass (DRM) and chlorophyll (CL) of *O. basilicum* 'genovese' as a function of different concentrations of potassium iodide.

Treatments (µM)	NL	NS	LS (mm)	FMS (g)
0	24.69± 1.03 <sup>a*</sup>	0.75± 0.96 <sup>a</sup>	76.86± 10.60 <sup>a</sup>	4.90± 0.37 <sup>a</sup>
25	20.56± 6.20 <sup>ab</sup>	0.25± 0.50 <sup>a</sup>	74.30± 21.03 <sup>a</sup>	2.19± 1.05 <sup>b</sup>
50	14.53± 2.69 <sup>b</sup>	1.00± 0.00 <sup>a</sup>	79.91± 14.14 <sup>a</sup>	2.15± 0.79 <sup>b</sup>
75	13.89± 4.66 <sup>b</sup>	0.25± 0.50 <sup>a</sup>	59.66± 6.56 <sup>a</sup>	2.48± 0.29 <sup>b</sup>
100	15.57± 3.56 <sup>b</sup>	0.50± 1.00 <sup>a</sup>	75.24± 6.32 <sup>a</sup>	2.70± 0.46 <sup>b</sup>

Treatments (µM)	DMS (g)	FRM (g)	DMR (g)	CL
0	0.37± 0.04 <sup>a</sup>	3.86± 0.82 <sup>a</sup>	0.15± 0.03 <sup>a</sup>	19.53± 2.47 <sup>a</sup>
25	0.27± 0.12 <sup>a</sup>	3.12± 1.36 <sup>a</sup>	0.12± 0.04 <sup>a</sup>	22.51± 1.87 <sup>a</sup>
50	0.29± 0.17 <sup>a</sup>	2.82± 0.99 <sup>a</sup>	0.11± 0.03 <sup>a</sup>	23.32± 1.91 <sup>a</sup>
75	0.22± 0.05 <sup>a</sup>	2.23± 0.68 <sup>a</sup>	0.09± 0.02 <sup>a</sup>	22.78± 0.89 <sup>a</sup>
100	0.19± 0.04 <sup>a</sup>	2.00± 0.72 <sup>a</sup>	0.09± 0.04 <sup>a</sup>	23.30± 3.40 <sup>a</sup>

\*Means followed by the same letter in the column do not differ by Tukey's test 5%

**Table 6.** Nitrogen (N), potassium (K), Calcium (Ca) and Magnesium (Mg) measured at the end of 80 days of shoots (S) and roots (R) of *O. basilicum* 'genovese' as a function of different concentrations of potassium iodide.

Treatments (µM)	g.Kg <sup>-1</sup>							
	N		K		Ca		Mg	
	S	R	S	R	S	R	S	R
0	48.74±4.18 <sup>a*</sup>	38.81±0.59 <sup>b</sup>	15.13±4.75 <sup>a</sup>	12.19±1.78 <sup>a</sup>	3.16±0.43 <sup>a</sup>	1.44±0.21 <sup>a</sup>	0.38±0.05 <sup>a</sup>	0.27±0.01 <sup>a</sup>

25	41.14±2.06 <sup>a</sup>	38.52±1.47 <sup>b</sup>	10.51±0.59 <sup>a</sup>	10.93±3.56 <sup>a</sup>	3.17±0.03 <sup>a</sup>	1.73±0.12 <sup>a</sup>	0.33±0.0 <sup>a</sup>	0.25±0.07 <sup>a</sup>
50	44.30±1.89 <sup>a</sup>	46.18±1.52 <sup>a</sup>	14.30±0.0 <sup>a</sup>	10.51±1.78 <sup>a</sup>	3.90±0.11 <sup>a</sup>	1.79±0.20 <sup>a</sup>	0.41±0.05 <sup>a</sup>	0.30±0.03 <sup>a</sup>
75	45.04±0.54 <sup>a</sup>	44.55±1.32 <sup>a</sup>	14.71±0.59 <sup>a</sup>	10.51±0.59 <sup>a</sup>	3.54±0.31 <sup>a</sup>	1.90±0.98 <sup>a</sup>	0.38±0.017 <sup>a</sup>	0.35±0.03 <sup>a</sup>
100	46.47±6.78 <sup>a</sup>	47.95±1.10 <sup>a</sup>	14.71±0.59 <sup>a</sup>	13.03±5.35 <sup>a</sup>	3.71±0.07 <sup>a</sup>	2.24±0.49 <sup>a</sup>	0.43±0.035 <sup>a</sup>	0.44±0.15 <sup>a</sup>

\* Means followed by the same letter in the column do not differ by Tukey's test 5%.

**Table 7.** Total antioxidant activity from 2,2-difenil-1-picrilhidrazil (DPPH) obtained after 80 days of fresh leaves *O. basilicum* 'genovese' as a function of different concentrations of potassium iodide.

Treatments ( $\mu\text{M}$ )	Total antioxidant activity (%)
0	14.43 ±0.15 <sup>a</sup> *
25	17.26±2.68 <sup>a</sup>
50	14.95±0.78 <sup>a</sup>
75	13.6±1.73 <sup>a</sup>
100	17.11±1.34 <sup>a</sup>

\* Means followed by the same letter in the column do not differ by Tukey's test 5%

were performed in triplicates (515 nm) until stabilization (30 in 30 min) using a Beckman 640 B spectrophotometer. Results are expressed as a percentage of free radical sequestration (% SRL), according to the following equation:

$$\%SRL = \frac{(CA-AS)}{CA} \times 100$$

Where; Ca = control absorbance, and  
As = absorbance of the sample.

#### Enzyme analyses

Plant extract was produced using 200 mg of fresh leaves macerated in liquid nitrogen (Bonacina et al., 2017) to measure three antioxidant enzymes SOD (EC 1.15.1.1) was measured by inhibition of the photoreduction of nitroblue tetrazolium (NBT) and was expressed as U SOD  $\text{g}^{-1}\text{MF}$  per min (Giannopolitis and Ries, 1977); CAT (EC 1.11.1.6) was measured according to the protocol of Havir and McHale (1987) using the molar extinction coefficient of  $36 \text{ M}^{-1}$  per min and expressed in  $\text{mmol H}_2\text{O}_2 \text{ g}^{-1} \text{MF}$  per min (Anderson et al., 1995); and APX (EC 1.11.1.11) activity was measured according to the protocol of de Nakano and Asada (1981) by the degradation of  $\text{H}_2\text{O}_2$  to 290 nm within 1 min, quantified using the molar extinction coefficient of  $2.8 \text{ mM}^{-1}$  per cm and was expressed as  $\text{mmol ascorbic acid g}^{-1} \text{MF}$  per min. Analyses of both experiments were performed after 80 days. Enzyme activity was measured using flat-bottomed 96-well plates and with a UV-VIS spectrophotometer (Espectra Max Plus<sup>®</sup> San Jose, US) with the software SoftMax Pro 6.5.1 (Molecular Devices<sup>®</sup> San Jose, US).

#### Statistical analyses

Both experiments were set up in a completely randomized design with five replicates, and each replicate comprised six flasks with four seeds. Normal distribution of biometric data and antioxidant activity was tested using a Shapiro-Wilk test. Data that significantly deviated from normal distribution were subjected to a Kruskal-Wallis test using the software Assisat version 7.7 (Silva and Azevedo, 2016). All other data were tested using an analysis of variance, and means were compared using a Tukey's test with the software SISVAR 5.6 (Ferreira, 2011). Statistical significance is reported at  $p \leq 0.05$ .

#### Conclusions

Growth of seedlings of *O. basilicum* 'Genovese' was stable at photoperiods of 16 h and 24 h, however, growth regulators exerted a beneficial effect. Antioxidant defense mechanisms were important to ameliorate adverse effects. Antioxidant activity and APX concentrations produced higher average values in the treatments with 16 h light and with addition of growth regulators.

Additional K supplementation showed no beneficial effect on growth but led to a decrease in the number of leaves and in shoot mass. CAT showed higher activity at higher KI concentrations, therefore enzymatic mechanisms seem to be important for the control of free radicals since antioxidant activity did not differ between treatments.

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