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RESEARCH NOTE

Strategy for suppressing redox stress during tomato (*Solanum lycopersicum L.)* seed germination

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Abstract

P.M. Arce-Amezquita, K. Max-Rodriguez, A.C. Rosales-Niebla, and F.H. Ruiz-Espinoza. 2019. Strategy for suppressing redox stress during tomato (*Solanum lycopersicum*) seed germination. Cien. Inv. Agr. 46(3): 286-294. A series of pyrimidinol-based compounds have been synthesized and evaluated for their ability to increase seedling biomass during seedling development. The compounds have the ability to reduce oxidative stress catalytically as natural phenolic antioxidants such as α-tocopherol do, but more efficiently. As a result, a compound with an appropriate structure (lipophilicity) effectively reduces the oxidative stress generated during seedling development, thus increasing biomass accumulation. Tomato seeds were pretreated with the synthesized antioxidants **Py1C**, **Py8C** and **Py12C** using acetone as the vehicle for application. None of the analogs tested affected total germination or hypocotyl dry biomass; however, seed treatment with **Py8C** and **Py12C** successfully increased radicle dry biomass by, on average, 44 % and 88 %, respectively. Compounds with these properties may be useful for the production of seedlings with enhanced characteristics such as vigor and stress resistance.

Keywords: Antioxidants, dry biomass, germination, oxidative stress, pyrimidinol.

Introduction

Oxidative stress is linked to the electron transport chain in the membranes of mitochondria and chloroplasts during ATP production. Therefore, numerous energetically demanding physiological processes, such as germination and seedling development, are associated with oxidative stress (Bailly, 2004). It is well known that mitochondria are the principal subcellular compartments of oxygen consumption and the principal source of reactive oxygen species (ROS) (Puntarulo *et al.*, 1988). Most of the ATP required for the early stages of development comes from mitochondria; therefore, the generation of ROS during this process is mainly associated with cellular respiration. At later stages of development, photophosphorylation in chloroplasts becomes the main source of ATP to cover the energy requirements of proper plant development; then, chloroplasts also become a primary source of ROS (Asada, 2006). Plants are equipped with a series of proteins including

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superoxide dismutase, catalase and glutathione peroxidase that mitigate the formation of ROS during ATP production. However, during higher oxidative stress, overproduction of ROS exceeds the detoxification capacity of these proteins, causing damage to important biomolecules such as lipids, proteins and nucleic acids (Dat *et al*., 2000). To protect these important biomolecules from damage, plants also contain small molecules such as ascorbic acid (vitamin C) and phenolic compounds such as α-tocopherol (vitamin E), flavonoids, tannins and phenolic acids that play an important role in oxidative damage mitigation. The production (qualitative and quantitative) and performance of these compounds in the plant are significantly influenced by the environmental (Campos-Vargas *et al*., 2012; Gutiérrez-Tlahque *et al*., 2018) and nutritional (Yañez-Mansilla *et al*., 2014) conditions where the plant grows and develops. These small molecules are distributed within cells according to physicochemical properties such as lipophilicity. These proteins and small molecules are considered the antioxidant defense mechanism in cells. However, when the damage to biomolecules cannot be mitigated and is too extensive, cells become apoptotic (Lenaz *et al*., 1998).

The correct functioning of mitochondria and chloroplasts, mainly the electron transport chain, is primarily attributable to the integrity of the biomolecules that form them. These two organelles are of great interest for plant health. One strategy for achieving the correct functioning of mitochondria and chloroplasts is the application of substances with antioxidant activities greater than those of natural phenolic antioxidants, in order to increase seedling antioxidant defenses (Arce *et al*., 2011). Recently, pyrimidinol-based antioxidants have been demonstrated to be more stable and effective than phenolic antioxidants due to their structural characteristics. The presence of an amino group at the *para* position to the hydroxyl group and of nitrogen atoms as part of the aromatic ring gives these compounds lower oxygen-hydrogen bond dissociation energy and higher ionization potentials (Pratt *et al*., 2001; Valgimigli *et al*., 2003; Wijtmans *et al*., 2003). This particular arrangement of atoms gives them higher ROS-inhibition ability and more stability to air than those of phenolic antioxidants (Arce *et al*., 2012; Khdour *et al*., 2013).

The application of substances with such properties might protect important biomolecules and support ATP production, thereby maintaining the maximum efficiency of cellular division in order to accumulate biomass. Therefore, the objective of this work was the preparation and evaluation of the effects of **Py1C** and two new pyrimidinol-based compounds with enhanced antioxidant properties on tomato seed germination. Moreover, these compounds were evaluated for their effect on dry biomass accumulation as an indirect indication of cytoprotection against oxidative stress.

Materials and methods

Synthesis

All reagents and solvents used herein were purchased from Sigma-Aldrich and were used without further purification. Column chromatography was carried out using silica gel (60 Å particle size, 230–240 mesh). Analytical thin layer chromatography (TLC) separations were carried out on glass plates coated with silica gel (60 Å particle size F254). The TLC chromatograms were developed using UV irradiation or by immersing the plates in ceric ammonium molybdate staining solution followed by heating. Melting points were recorded on a MelTemp apparatus.

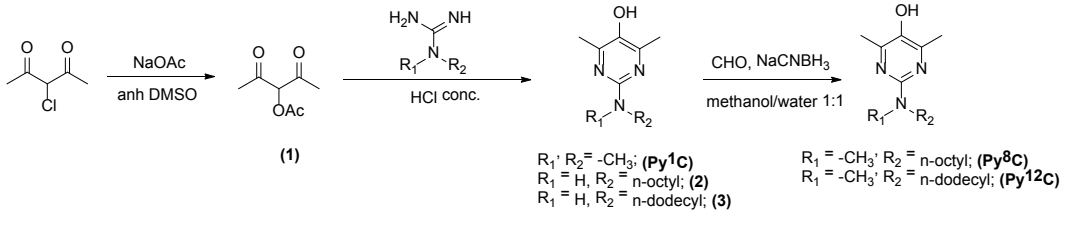
To synthesize the pyrimidinol antioxidants used in this experiment, a short pathway was developed. The strategy consisted of the addition of a guanidine compound bearing different size alkyl chains to 3-acetoxy-2,4-pentanodione, which was prepared by reacting 3-chloro-2,4-pentanodione with sodium acetate. **Py1C** was obtained in one

step using *N.N′*-dimethylguanidine. However, to obtain analogs **Py8C** and **Py12C**, further reductive methylation was performed after condensation with the corresponding guanidine compound (scheme 1).

3-acetoxy-2,4-pentanodione (**1**). To a stirred mixture containing 7.25 g (88.4 mmol) of sodium acetate in 30 mL of anhydrous dimethyl sulfoxide in a round-bottomed flask, 5.00 mL (5.95 g; 44.2 mmol) of 3-chloro-2,4-pentanodione was added. The reaction mixture was stirred at 25 °C for 3 h under nitrogen atmosphere using a magnetic stirrer (VWR®). The reaction mixture was diluted with 200 mL of distilled water and extracted with 300 mL of ethyl acetate in a separatory funnel. The organic solution was then washed with 200 mL of brine in a separatory funnel, dried over anhydrous Na_2SO_4 and then concentrated under diminished pressure in a rotary evaporator (BUCHI®, R-114). The residue was purified by chromatography on a silica gel column. Elution was performed with an ethyl acetate/hexanes 1:4 mixture. After evaporation of the fractions containing the expected compound in a rotary evaporator, the product was obtained as an orange liquid; the yield was 4.36 g (62 %). Silica gel TLC R*f* = 0.32 using a 9:1 hexane/ethyl acetate mixture as the mobile phase.

2-(dimethylamino)-4,6-dimethylpyrimidin-5-ol (**Py1C**) (Nara *et al*., 2008). To a stirred solution containing 1.00 g (3.67 mmol) of 1,1-dimethylguanidine hydrosulfate in 20 mL of 12N HCl in a round-bottomed flask, 2.02 g (12.8 mmol) of 3-acetoxy-2,4-pentanodione was added in three portions every 30 min. The reaction mixture was then stirred at 25 °C for 16 h using a magnetic stirrer (VWR®). The reaction mixture was neutralized with solid Na_2CO_3 and then extracted with three 50 mL portions of ethyl acetate in a separatory funnel. The combined organic mixture was washed with 100 mL of brine in a separatory funnel, dried over anhydrous Na_2SO_4 and then concentrated under diminished pressure in a rotary evaporator (BUCHI®, R-114). The residue was purified by chromatography on a silica gel column. Elution with an ethyl acetate/hexanes/methanol 16:3:1 mixture gave the expected compound as a yellowish solid; the yield 109 was mg (18 %) and the mp = 143-146 °C. Silica gel TLC R*f* = 0.20 using a 16:3:1 ethyl acetate/hexanes/methanol mixture as the mobile phase.

4,6-dimethyl-2-(octylamino)pyrimidin-5-ol (**2**). To a stirred solution containing 1.00 g (4.54 mmol) of 1-octhylguanidine hydrosulfate in 36 mL of 12N HCl in a round-bottomed flask, 2.51 g (15.9 mmol) 3-acetoxy-2,4-pentanodione was added in three portions every 30 min. The reaction mixture was then stirred at 25 °C for 16 h using a magnetic stirrer (VWR®). The reaction mixture was neutralized with solid Na_2CO_3 and then extracted with three 50 mL portions of ethyl acetate in a separatory funnel. The combined organic mixture was washed with 100 mL of brine in a separatory funnel, dried over anhydrous Na_2SO_4 and then concentrated under diminished pressure in a rotary evaporator (BUCHI®, R-114). The residue was purified by chromatography on a silica gel column. Elution with an ethyl acetate/hexane/methanol 16:3:1 mixture gave the expected compound as a yellowish solid; the yield was 282 mg (25 %) and mp = 50-52 °C. Silica gel TLC



Scheme 1. Strategy followed for the synthesis of **Py1C**, **Py8C** and **Py12C**

R*f* = 0.25 using a 16:3:1 ethyl acetate/hexane/methanol mixture as the mobile phase.

2-(dodecylamino)-4,6-dimethylpyrimidin-5-ol (**3**). To a stirred solution containing 250 mg (0.87 mmol) of n-dodecylguanidine acetate in 5.00 mL of 12N HCl in a round-bottomed flask, 550 mg (3.48 mmol) of 3-acetoxy-2,4-pentanodione was added in three portions every 30 min. The reaction mixture was then stirred at 25 °C for 16 h using a magnetic stirrer (VWR®). The reaction mixture was neutralized with solid Na_2CO_3 and then extracted with three 50 mL portions of ethyl acetate in a separatory funnel. The combined organic mixture was washed with 100 mL of brine in a separatory funnel, dried over anhydrous Na_2SO_4 and then concentrated under diminished pressure in a rotary evaporator (BUCHI®, R-114). The residue was purified by chromatography on a silica gel column. Elution with an ethyl acetate/hexane 2:3 mixture gave the expected compound as a yellowish solid; the yield was 41.0 mg (15 %) and mp = 36-38 °C. Silica gel TLC R*f* = 0.25 using a 2:3 ethyl acetate/hexane mixture as the mobile phase.

4,6-dimethyl-2-(methyl(octyl)amino)pyrimidin-5-ol (**Py8C**). To a stirred solution containing 268 mg (1.07 mmol) of 4,6-dimethyl-2-(octylamino) pyrimidin-5-ol in 10 mL of methanol/formalin 1:1 in a round-bottomed flask, 269 mg (4.28 mmol) of sodium cyanoborohydride was added. The reaction mixture was then stirred at 25 °C for 3 h using a magnetic stirrer (VWR®). The reaction mixture was quenched with glacial acetic acid and then extracted with three 50 mL portions of ethyl acetate in a separatory funnel. The combined organic mixture was washed with 100 mL of brine in a separatory funnel, dried over anhydrous Na_2SO_4 and then concentrated under diminished pressure in a rotary evaporator (BUCHI®, R-114). The residue was purified by chromatography on a silica gel column. Elution with ethyl acetate/hexanes/methanol 16:3:1 mixture gave the expected compound as a yellowish solid; the yield was 121 mg (57 %) and mp = 34-36 °C. Silica gel TLC R*f* = 0.45 using a 16:3:1 ethyl acetate/hexane/methanol mixture as the mobile phase.

2-(dodecyl(methyl)amino)-4,6-dimethylpyrimidin-5-ol (**Py12C**). To a stirred solution containing 90 mg (0.29 mmol) of 2-(dodecylamino)-4,6-dimethylpyrimidin-5-ol in 4 mL methanol/formalin 1:1 in a round-bottomed flask, 72 mg (1.16 mmol) of sodium cyanoborohydride was added. The reaction mixture was then stirred at 25 °C for 3 h using a magnetic stirrer (VWR®). The reaction mixture was quenched with glacial acetic acid and then extracted with three 50 mL portions of ethyl acetate in a separatory funnel. The combined organic mixture was washed with 100 mL of brine in a separatory funnel, dried over anhydrous Na_2SO_4 and then concentrated under diminished pressure in a rotary evaporator (BUCHI®, R-114). The residue was purified by chromatography on a silica gel column. Elution with an ethyl acetate/hexane 1:9 mixture gave the expected compound as a yellowish solid; the yield was 47 mg (50 %) and mp = 45-47 °C. Silica gel TLC R*f* = 0.25 using a 1:9 ethyl acetate/hexane mixture as the mobile phase.

Germination test

Py1C, **Py8C** and **Py12C** were then evaluated in tomato seed germination. For the germination test, the seeds were pretreated by soaking them in acetone containing pyrimidinols **Py1C**, **Py8C** and **Py12C** at 200 and 1000 μM concentrations for 24 h. Per each gram of seeds, 30 mL of the respective solutions were used. The controls were untreated seeds and seeds pretreated with pure acetone. Seeds were filtered and dried in an oven at 40 °C for 30 min under diminished pressure before the test. The experiment was set in a completely random design that consisted of four replicates of 25 seeds per treatment and controls. To avoid disturbances in germination due to rehumidification in case of desiccation during the experiment, the seeds were placed

on four filter paper discs (Whatman™ No. 1) at once in 9 cm-diameter plastic Petri dishes containing 10 mL of distilled water. The dishes with the seeds were kept at 25 °C for 7 days in a dark germination chamber. Seeds with testa rupture were considered germinated. The obtained seedlings were separated by hypocotyl and radicle and then dried separately in an oven at 100 °C for 48 h. The dried material was weighed in an analytical balance (Mettler Toledo®, AG245) to obtain hypocotyl and radicle dry biomass. One-way univariate analysis of variance (ANOVA) was used to analyze the data obtained, and seed treatment was used as a fixed factor. The difference between the means was determined by the Tukey-HSD multiple range test at $P = 0.05$. Mean values were considered significantly different when $P \leq 0.05$. Statistical analysis was carried out using Statistica® v. 10.0.

Results

The compounds **Py1C, Py8C and Py12C** were obtained with 11 %, 8.8 % and 4.7 % overall yield, respectively.

None of the treatments significantly affected total germination, which was approximately 70 % in the treatments and in the controls that consisted of untreated seeds and seeds soaked with pure acetone (Figure 1).

The seedlings obtained did not show a significant difference in average hypocotyl dry biomass per seedling obtained from seeds treated with analogs **Py1C**, **Py8C** and **Py12C** compared to the controls after 7 days of germination (Figure 2). The average hypocotyl dry biomass per seedling was approximately 1.1 mg/hypocotyl.

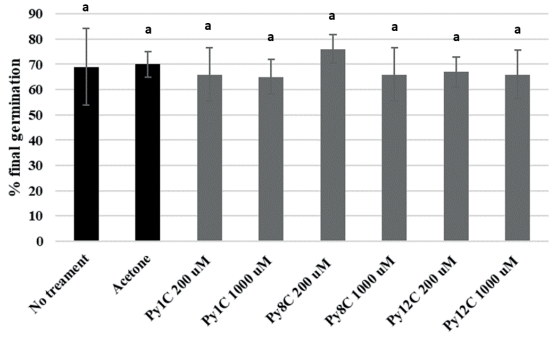


Figure 1. Effect of seed pretreatment with pyrimidinol antioxidants on final germination at 7 days. Values are means ± SDs; different letters indicate significant differences at $P < 0.05$.

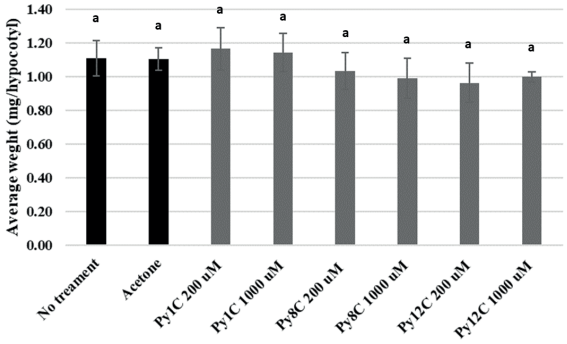


Figure 2. Effect of seed pretreatment with different concentrations of pyrimidinol antioxidants on hypocotyl dry biomass. Values are means ± SDs; different letters indicate significant differences at $P < 0.05$.

However, the average radicle dry biomass per seedling was significantly affected; analog **Py8C** increased it, on average, by 44 % and analog **Py12C**, by 88 % compared to those of the controls (Figure 3). Seedlings from the controls presented an average radicle dry biomass per seedling of approximately 0.09 mg/radicle, seedlings treated with **Py8C** presented an average radicle dry biomass per seedling of approximately 0.13 mg/ radicle and seedlings treated with **Py12C** presented an average radicle dry biomass per seedling of approximately 0.17 mg/radicle.

Seeds treated with the antioxidant **Py12C** showed exceptionally increased hypocotyl and radicle size compared to those of the controls among the seedlings obtained after seven days of germination in the dark at 25 °C, as shown in Figure 4.

Discussion

In an effort to obtain potent antioxidants that could be used for quenching oxidative stress, a series of three pyrimidinol analogs were synthesized. These compounds contain different-sized alkyl side chains that provide different pharmacokinetic properties in terms of lipophilicity.

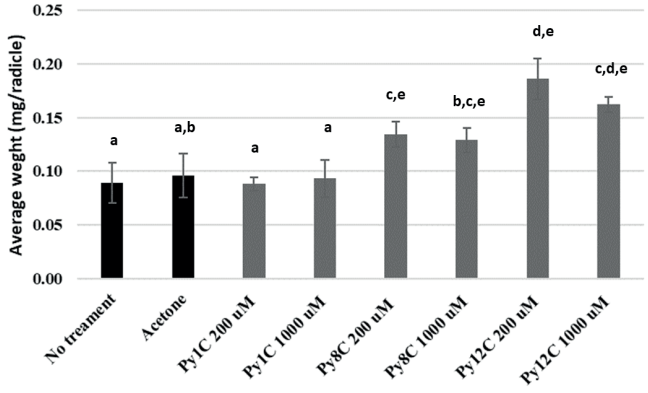


Figure 3. Effect of seed pretreatment with pyrimidinol antioxidants on radicle dry biomass. Values are means ± SDs; different letters indicate significant differences at $P < 0.05$.

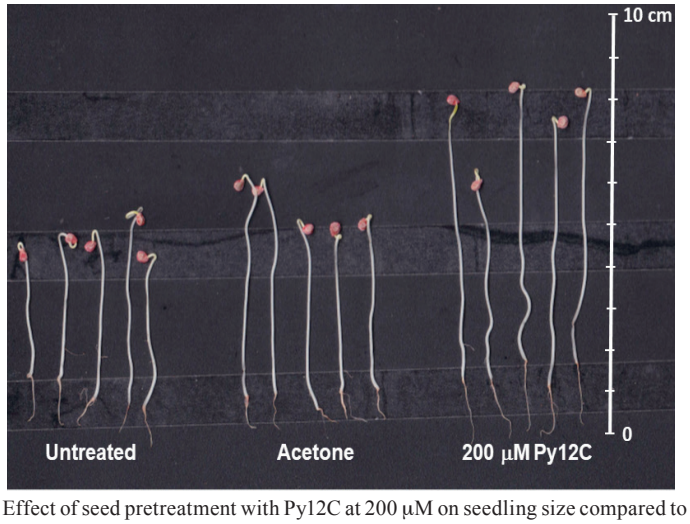


Figure 4. Effect of seed pretreatment with Py12C at 200 μM on seedling size compared to controls.

The antioxidant effect of these new pyrimidinol analogs was tested for the augmentation of biomass during the germination of tomato seeds. Interestingly, all three analogs have the same redox core, where the antioxidant chemistry happens; however, only compounds **Py8C** and **Py12C** showed an important effect on radicle dry biomass (Figure 3). Hypocotyl dry biomass was not affected after treatment with the antioxidants synthesized herein probably because there is an overproduction of ROS in radicles compared to that in hypocotyls; therefore, the antioxidant activity in radicles is more evident. This enhanced antioxidant activity is attributed to the fact that the compounds have the proper characteristics (possibly lipophilicity) that allow them to reach the mitochondria, which are the main source of oxidative stress during germination. Additionally, **Py1C** did not show any effect at all, probably due to the lack of uptake of this compound by the cells. These results suggest that **Py8C** and **Py12C** the cells by diffusion through the membranes.

Additionally, this series of antioxidant structures is based on a pyrimidinol moiety, which means that they are stable to air oxidation and have higher rates of oxidative stress quenching than phenol antioxidants, so only a few molecules need to reach the site of ROS generation in order to reduce oxidative stress. In addition, it is believed that antioxidants of this nature act catalytically, possibly being regenerated by vitamin C and the cofactor nicotine adenine dinucleotide (NADH) each time they are oxidized after quenching ROS (Liebler, 1993).

Previously, it has been shown that pretreatment of tomato seeds with natural antioxidants (b-carotene, ascorbic acid and lycopene) and the synthetic antioxidant Ambiol™ have positive effects on the shoot and root dry biomass of seedlings under drought stress. These positive effects were also observed in terms of leaf area and photosynthetic activity (Macdonald *et al*., 2008; Macdonald *et al*., 2009). Ascorbic acid has also been shown to increase dry biomass and drought resistance in wheat upon seed preconditioning (Farooq *et al*., 2013). Interestingly, the positive effects obtained after seed pretreatment with Ambiol™ are passed on to the next generations, even improving the production yield (Macdonald *et al*., 2010). Until now, aqueous solutions of antioxidants have been used for seed pretreatment, which meant that the germination process started during the treatment. In this experiment, we explored the possibility of using an organic solvent as a vehicle for the application of antioxidants. This strategy has been used previously for the application of organic compounds to dry seeds without affecting germination (Tao and Khan, 1974; O'Neill *et al*., 1979). Among the organic solvents, acetone has shown more compatibility with plant physiological conditions (Murphy, 1985). An important feature of using organic solvents is that the germination mechanism is not activated, allowing the possibility of sowing the seeds even months after the treatment (Khan *et al*., 1973). Another important aspect of using organic solvents is that compounds that typically cannot be utilized because of their poor water solubility can be applied to the seeds.

An interesting observation was the augmented size of seedlings grown from seeds treated with **Py12C** (Figure 4). The observed larger size correlates with the augmentation of radicle biomass; on the other hand, the hypocotyl seemed to only be elongated. Even though the hypocotyls were longer under the Py12C treatment, they had the same biomass as those in the controls. This phenomenon suggests changes in the absorption and use of water. The application of exogenous molecules that increase vigor and resistance by alleviating oxidative stress could be an important strategy for obtaining more substantial benefits from a given crop.

The main conclusions are as follows. Two new pyrimidinol antioxidants (**Py8C** and **Py12C**) were synthesized along with **Py1C. Py8C** and **Py12C** showed the ability to increase seedling radicle biomass upon application to dry seeds using acetone as the vehicle for application.

However, **Py12C** was shown to be more effective than **Py8C** and **Py1C**, and the latter did not show any activity in experiments carried out in this work. These findings suggest the importance of the antioxidant chemical structure in obtaining the most appropriate compounds for application in agronomy. The tendency toward more biological activity as the lipophilicity of the side chain increases requires the preparation and evaluation of more analogs with longer alkyl side chains. The utilization of techniques for the reduction in oxidative stress during important biochemical processes for plant establishment, such as the technique presented in this investigation, ensures the maximum efficiency of energy production and utilization. Thus, antioxidants such as pyrimidinol compounds could enhance plant resistance and vigor by facilitating the proper deployment of energy (ATP) for processes such as cellular division and plant defense against biotic and abiotic stress rather than using that energy to handle damage produced by ROS.

Resumen

P.M. Arce-Amezquita, K. Max-Rodriguez, A.C. Rosales-Niebla, y F.H. Ruiz-Espinoza. 2019. Estrategia para suprimir el estrés oxidativo durante la germinación de tomate (*Solanum lycopersicum*). Cien. Inv. Agr. 46(3): 286-294. Una serie de compuestos basados en pirimidinoles ha sido sintetizada y evaluada en el incremento de biomasa durante desarrollo de plántulas. Estos compuestos tienen la habilidad de reducir el estrés oxidativo catalíticamente como lo hacen los compuestos antioxidantes fenólicos naturales, como el α-tocoferol. Como resultado, un compuesto con la estructura apropiada (lipofilicidad) puede reducir eficientemente el estrés oxidativo generado durante el desarrollo de plántulas y, por consiguiente, incrementar la acumulación de biomasa. Semillas de tomate fueron pre-tratadas con antioxidantes sintetizados **Py1C**, **Py8C** y **Py12C** usando acetona como vehículo de aplicación. Ninguno de los análogos aplicados afectó la germinación total ni la biomasa seca de hipocótilo; sin embargo, el tratamiento de semillas con **Py8C** y **Py12C** incrementó satisfactoriamente en promedio 44 % y 88 % la biomasa seca de raíz, respectivamente. Compuestos con estas propiedades pueden ser útiles en la producción de plántulas con características mejoradas como vigor y resistencia al estrés.

Palabras clave: Antioxidantes, biomasa seca, estrés oxidativo, germinación, pirimidinol.

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