

Putrescine Biosynthesis Inhibition in Tomato by DFMA and DFMO Treatment

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[Abstract] This protocol can be used to inhibit the biosynthesis of polyamines, specifically putrescine, in tomato plants grown with NH_4^+ as a solely N source. In general, polyamines are positively charged small metabolites implicated in physiological processes, including organogenesis, embryogenesis, floral initiation and development, leaf senescence, pollen tube growth, fruit development and ripening and participate in the response to abiotic and biotic stresses (Tiburcio *et al.*, 2014). Polyamines are synthesized from amino acids by decarboxylation of ornithine or arginine by ornithine decarboxylase (ODC) or arginine decarboxylase (ADC), respectively (Walters, 2003). Tomato plants grown with NH_4^+ as the sole N source presented an increase of putrescine content in leaves (Fernández-Crespo *et al.*, 2015). To assess the importance of putrescine accumulation, DL- α -(Difluoromethyl)arginine (DFMA) and DL- α -(Difluoromethyl)ornithine (DFMO), inhibitors of putrescine synthesis, were used as irreversible inhibitors of ADC and ODC enzymes, respectively (Fallon and Phillips, 1988), with the purpose of reducing cellular putrescine accumulation induced by NH_4^+ nutrition.

The inhibitor solution containing 2 mM DFMA and 5 mM DFMO was applied directly to each pot during the week prior to sample collection. Putrescine content was reduced by 35.3% in tomato plants grown with NH_4^+ .

[Background] The application of the inhibitors DFMA and DFMO was normally performed in MS medium and *in vitro* assays (Perez-Amador *et al.*, 2002; Stes *et al.*, 2011). However, we needed to test effectiveness of these inhibitors *in vivo* with the purpose to maintain natural growth conditions. Moschou *et al.* (2008) demonstrated the inhibition effect of DFMA and DFMO when applied in hydroponic cultures at 0.1 mM and 1 mM respectively. In this work, we used similar approaches with some modifications: the hydroponic culture was changed by vermiculite growing medium and the concentration applied for the inhibitors was modified.

Materials and Reagents

1. Tomato seeds (*Solanum lycopersicum* Mill. cv. Ailsa Craig)
2. Vermiculite (Asfaltex SA, TERMITA®)
3. Potassium hydroxide (KOH) (Scharlab, catalog number: PO0275)
4. Potassium sulfate (K_2SO_4) (Scharlab, catalog number: PO0365)

5. Ortho-Phosphoric acid (H_3PO_4) (Scharlab, catalog number: AC1100)
6. Ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$ (Avantor Performance Materials, J.T.Baker, catalog number: 4628)
7. Calcium sulfate dihydrate ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$) (Scharlab, catalog number: CA0285)
8. Magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) (Scharlab, catalog number: MA0085)
9. Boric acid (H_3BO_3) (AppliChem, catalog number: 131015)
10. Manganese(II) sulfate monohydrate (MnSO_4) (Scharlab, catalog number: MA0131)
11. Zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) (Scharlab, catalog number: CI0207)
12. Copper(II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) (Scharlab, catalog number: CO0101)
13. Molybdenum trioxide (MoO_3) (Panreac, Vidrafoc, catalog number: 142791)
14. Sequestrene (Fe 6%) (Syngenta)
15. 2-(N-Morpholino) ethanesulfonic acid sodium salt (MES sodium salt) (Sigma-Aldrich, catalog number: M3885)
16. DL- α -(Difluoromethyl)arginine (DFMA) (Santa Cruz Biotechnology, catalog number: sc-211368)
17. DL- α -(Difluoromethyl)ornithine hydrochloride (DFMO) (Santa Cruz Biotechnology, catalog number: sc-252762)
18. Distilled water
19. Nutrient solution (see Recipes)
20. Inhibitors mix (see Recipes)

Equipment

1. Plant growth room (A.S.L Snijders)
2. Pots (50 ml) (Pöppelmann, model: Serie TO)
3. pH meter (HACH LANGE SPAIN, CRISON, model: GLP21)

Software

1. Statgraphics-plus software of Windows V.5 (Statistical Graphics Corp., Rockville, MD, USA)

Procedure

A. Tomato growth conditions

1. Tomato seeds (*Solanum lycopersicum* Mill. cv. Ailsa Craig) are germinated in pots (50 ml) with 100% vermiculite in a growth room under the following environmental conditions: light/dark cycle of 16/8 h, temperature of 24/18 °C, light intensity of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and relative humidity of 60%.
2. Seeds are irrigated twice a week with approximately 50 ml/pot of distilled water during 1 week.

3. Seedlings are irrigated twice each week for 3 weeks with approximately 50 ml/pot of nutrient solution. N is supplied in the form of $[(\text{NH}_4)_2\text{SO}_4]$ 0.33 g/L.
4. The pH of the nutrient solution is adjusted to 6.0 with 1 mM KOH.

B. Application of inhibitor mix to tomato plants

1. 20 uniform tomato plants grown as described above are divided into two groups (10 plants are not treated and 10 plants are treated with the inhibitor solution).
2. 21-day-old tomato plants are treated with 2 ml of the inhibitor mix by soil drench (final concentration in each pot is DMFA 80 nM and DFMO 200 nM)
3. 23-day-old tomato plants are treated with 4 ml of the inhibitor mix by soil drench (final concentration in each pot is DMFA 160 nM and DFMO 400 nM).
4. 25-day-old and 27-day-old tomato plants are treated with 8 ml of the inhibitor mix (final concentration in each pot is DMFA 320 nM and DFMO 800 nM) (Figure 1). Inhibitors mix treatment does not produce changes in tomato growth (Figure 2).
5. 24 h after the last treatment, the 3rd and 4th true leaves of treated or not treated tomato plants (Figure 3) are collected and stored at -80 °C.
6. A pool of 10 plants is crushed in liquid N₂ in a mortar and 0.2 g is further used for putrescine extraction.
7. Three biological replicated are realized.

C. Putrescine quantification

1. Putrescine quantification is realized following the protocol of Sanchez-Lopez *et al.* (2009) by ion pair LC coupled with electrospray tandem mass spectrometry.
2. Putrescine content is expressed as the average of three independent experiments (Fernández-Crespo *et al.*, 2015).

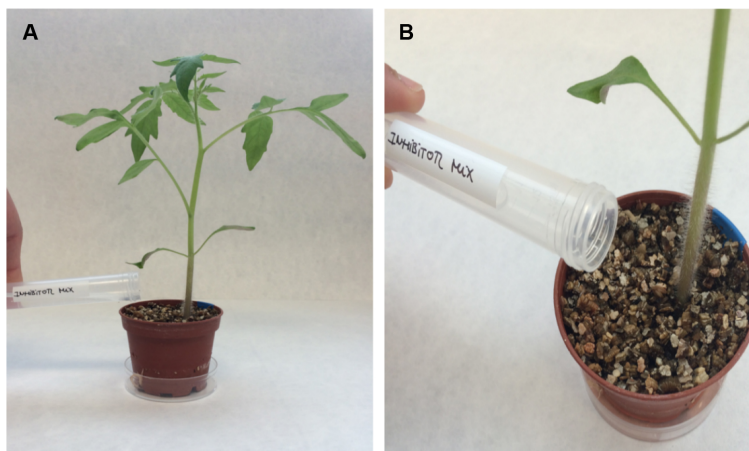


Figure 1. Application of inhibitor mix to tomato plants grown in vermiculite

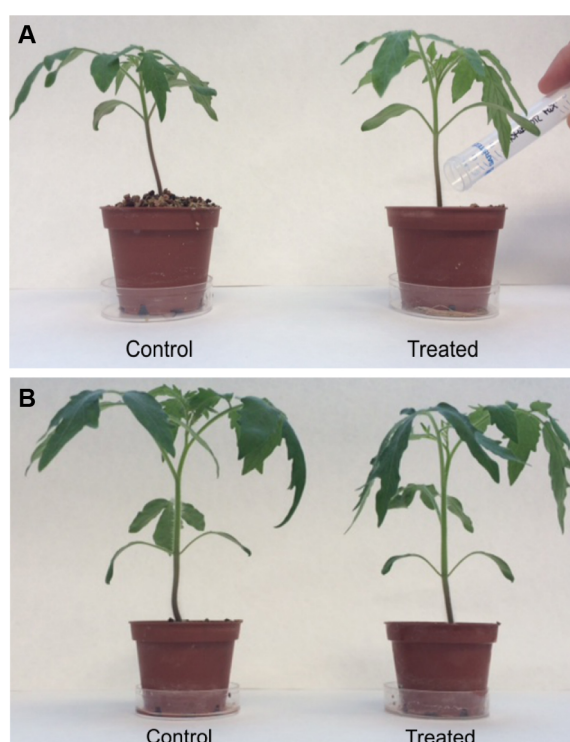


Figure 2. Tomato plants treated with inhibitor mix do not display changes in growth. A. Control and treated 21-day-old tomato plants; B. The same plants after one week of inhibitors treatment.

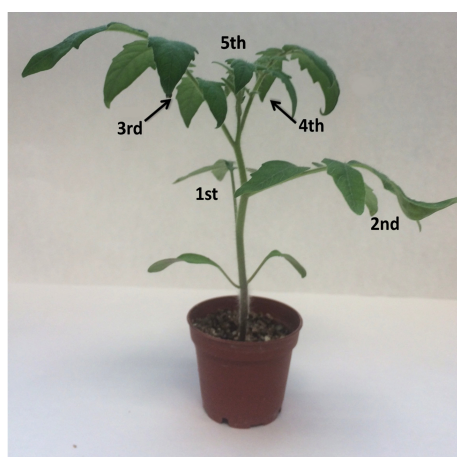


Figure 3. Tomato plant with five true leaves. The arrows indicate the 3rd and 4th true leaf of tomato plants collected for putrescine quantification.

Data analysis

Statistical analysis is carried out using a one-way analysis of variance in the Statgraphics-plus software of Windows V.5 (Statistical Graphics Corp., Rockville, MD, USA). The putrescine content

means are calculated and expressed with standard errors and compared using a Fisher's least-significant difference test at the 95% confidence interval. The experiment is repeated three times.

Notes

Fernández-Crespo *et al.* (2015) demonstrated that tomato plants grown with nutrient solution containing NH_4^+ 5 mM as a solely N source displayed high putrescine content compared with control plants, to which N was provided as NO_3^- form [(KNO_3 (4 mM) and $\text{Ca}(\text{NO}_3)_2$ (5 mM)]. Putrescine reduction (35.3%) by inhibitors application was tested in tomato plants grown with $(\text{NH}_4)_2\text{SO}_4$ as N source.

Recipes

1. Nutrient solution

0.33 g/L $(\text{NH}_4)_2\text{SO}_4$
0.35 g/L K_2SO_4
0.07 ml/L H_3PO_4
0.04 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
0.54 g/L $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$
2.86 mg/L H_3BO_3
2.2 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$
0.11 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
0.15 mg/L MnSO_4
0.09 mg/L MoO_3
6.7 mg/L sequestrene (6% Fe)
217 mg/L MES sodium salt
Adjust pH to 6.0 with 1 mM KOH

2. Inhibitors mix

DL- α -(Difluoromethyl)arginine DFMA (2 mM)
DL- α -(Difluoromethyl)ornithine hydrochloride DFMO (5 mM)
Both compounds were dissolved in distilled water.

Note: Both solutions (Nutrient solution and Inhibitors mix) do not need to be sterilized.

Acknowledgments

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