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Growth and metabolism of onion seedlings as affected by the application of humic substances, mycorrhizal inoculation and elevated CO_2

Marcelle M. Bettoni[a], Átila F. Mogor[a], Volnei Pauletti[a], Nieves Goicoechea[b,*]

[a] Departamento de Fitotecnia e Fitossanitarismo, Setor de Ciências Agrárias, Universidade Federal do Paraná, Rua dos Funcionários, 1540. Juvevê, Curitiba, Brazil

[b] Departamento de Biología Ambiental, Grupo de Fisiología del Estrés en Plantas (Unidad Asociada al CSIC, EEAD, Zaragoza e ICVV, Logroño), Facultades de Ciencias y Farmacia, University of Navarra, Irunlarrea 1, E-31008 Pamplona, Spain

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ABSTRACT

Onion (*Allium cepa* L.) is a crop with great economic importance over the world. The vigor of seedlings plays a crucial role in the posterior growth and quality of bulbs. Application of humic substances (HS), inoculation of arbuscular mycorrhizal fungi (AMF) in the soil and enhancement of atmospheric CO_2 are three factors that can influence plant growth and development. Therefore, our main objective was to assess the effect of each of the abovementioned factors, separately or interacting, on the metabolism and growth of onion seedlings before bulb formation and under greenhouse conditions. Results showed that these three factors appear as valid horticultural techniques for improving growth and quality of onion seedlings cultivated in greenhouse even when mycorrhizal colonization of roots does not achieve high rates. Beneficial effects of HS were additive to those of mycorrhizal inoculation or elevated CO_2 (ECO_2) on shoot and root biomass production. The triple interaction between exogenous HS application, mycorrhizal inoculation and ECO_2 induced the highest accumulation of soluble sugars, proteins and proline in leaves, suggesting that such interaction was the most effective for increasing the quality of onion shoots as source organs for posterior growth and quality of bulbs and also for enhancing the tolerance of onion seedlings to environmental stresses.

1. Introduction

Onion (*Allium cepa* L.) is a crop with great economic importance. It is the second most important vegetable crop in the world with approximately 4 million Ha of harvested area and about 80 million tons produced in 2012. Only in Brazil the production exceeded 1.5 million tons and in Spain production almost reached 1.2 million tons, being this last country one of the main producing countries of onions in the European Union (FAOSTAT, 2014).

The size of the onion bulb is dependent upon the number and size of the green leaves at the time of maturity. There is a ring of onion for every leaf and the larger the size of the leaf, the bigger will be the ring (Addai et al., 2014). Therefore, the vigor of seedlings plays a crucial role in the posterior growth and quality of bulbs. Humic substances (HS), arbuscular mycorrhizal fungi (AMF) and enhanced atmospheric CO_2 (ECO_2) are three factors that may strongly influence growth and metabolism of onion seedlings.

Humic substances (HS) have been included among the natural molecules that exert physiological influences on plant growth (Nardi et al., 2002). They are derived from the degradation and decomposition of dead biological material in soils and are considered as supramolecular associations of self-assembling heterogenous and relatively small molecules (Piccolo, 2001). It has been reported that the HS can affect plant physiology because they may exert hormone-like effects (Nardi et al., 2002; Zandonadi et al., 2007), influence photosynthesis (Ferretti et al., 1991), activate some enzymes (Vaughan et al., 1985), increase mineral availability

Abbreviations: ACO_2, ambient CO_2; AMF, arbuscular mycorrhizal fungi; BSA, bovine serum albumin; Chl, chlorophyll; DM, dry matter; DPPH, αα-Diphenil-β-picrylhydrazyl radical scavenging activity; E, extension of mycorrhizal colonization; EC, electrical conductivity; ECO_2, elevated CO_2; FA, fulvic acids; FW, fresh weight; HA, humic acids; HS, humic substances; 0HS, without application of humic substances; 20HS, with application of humic substances; I, incidence of mycorrhizal colonization; Int, intensity of mycorrhizal colonization; KPB, potassium phosphate buffer; L, mycorrhizal colonization in length; MEI, mycorrhizal efficiency index; +M, inoculated with mycorrhizal fungi; −M, non-inoculated with mycorrhizal fungi; TSP, total soluble proteins; TSS, total soluble sugars; W, mycorrhizal colonization in width; WC, water content.

* Corresponding author. Tel.: +34 948 425600x806489; fax: +34 948 425619.
E-mail address: niegoi@unav.es (N. Goicoechea).

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(Varanini and Pinton, 2001; Murillo et al., 2005; Eyheraguibel et al., 2008) and/or stimulate beneficial soil microorganisms (Linderman and Davis, 2001). Among HS, the fulvic acids (FA) appear to confer a specific bioactivity to HS because of their loose conformation and hydrophilic nature. In addition, due to their substantial structural flexibility and heteregenous composition, FA are likely to release mobile hormone-like molecules, which may enter the biological pathways related to auxin activities (Zancani et al., 2011).

Mycorrhizal fungi colonize the roots of over 80% of plant species mostly to the mutual benefit of both plant host and fungus (Smith and Read, 2008). The most common are the arbuscular mycorrhizas (AM), which are formed by the majority of crop and horticultural plants, including onion. The association of onion with AMF can enhance plant height, leaf area index, chlorophyll content, total plant biomass as well as bulb dry mass and diameter (Bolandnazar et al., 2007). In addition, mycorrhizal symbiosis can improve the defence responses of onion plants against bacterial and fungal pathogens due to increased chitinase activity (Dumas-Gaudot et al., 1992), higher antimicrobial activity and enhanced levels of phenolic compounds (Lokhandwala et al., 2014) in roots. Mycorrhizal association can also favour the nitrate uptake by onions cultivated on dry soils (Azcón and Tobar, 1998). However, the effectiveness of AM for improving nutrient uptake and yield of onions can vary according to the type of fungus inoculated to plants. Charron et al. (2001a,b) found that bulbs of onions inoculated with *Glomus versiforme* were firmer than those inoculated with *Rhizophagus intraradices* (formerly *G. intraradices*) and the P, N and Zn concentrations were higher in onion plants colonized by *G. versiforme* than in those colonized by *R. intraradices*. The markedly mycotrophic character of onions has led to use this culture to enrich soil with mycorrhizal propagules prior planting some fruit trees (Panja and Chaudhuri, 2004).

The increase of atmospheric CO_2 as a consequence of global change and/or horticultural practices affects plant growth and development. The enhanced CO_2 concentration (ECO_2) increases the potential net photosynthesis in C3 plants, such as onion (Drake et al., 1997) and therefore can improve yield over short-term exposures (Oliveira et al., 2010). In an assay performed under greenhouse conditions, Savé et al. (2007) concluded that CO_2 fertilization shows interesting perspectives to improve horticultural techniques in order to enhance plant medicinal productivity. However, when the synthesis of carbohydrates in plants exposed to ECO_2 exceeds the capacity to produce new sinks, the plants decrease their photosynthetic rate so as to balance source activity and sink capacity (Thomas and Strain, 1991). In onion, sink effect of bulbs may counteract at least in part the expected acclimation of photosynthesis over medium or long-term exposures to ECO_2 and the presence of AMF colonizing onion roots would presumably increase the sink effect.

The main objective of our study was to assess the effect of each of the abovementioned factors, application of humic substances (20HS), mycorrhizal inoculation (+M) and elevated CO_2 (ECO_2), separately or interacting, on the metabolism and growth of onion seedlings before bulb formation. Special attention was paid to the levels of photosynthetic pigments, sugars, proteins and proline in leaves as well as to acid phosphatase in roots. Levels of soluble phenolics and the total antioxidant capacity of leaves were also tested.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds from *Allium cepa* L. cv. Alfa São Francisco—Cycle VIII (Embrapa Semi-Árido) were germinated on a mixture of light peat and sand (1:1, v:v) (on 26th March 2013). Peat (Floragard, Vilassar de Mar, Barcelona, Spain) had a pH of 5.2–6.0, 70–150 mg L^{-1} of nitrogen, 80–180 mg L^{-1} P_2O_5 and 140–220 mg L^{-1} K_2O and it was previously sterilized at 100 °C for 1 h on three consecutive days. Twenty two days after sowing (on 17th April 2013), seedlings were transferred to trays filled with a mixture of vermiculite- siliceous sand- sterilized light peat (2.5:2.5:1, v:v:v). Each tray had 60 cells with a capacity of 60 mL each one. The experiment was conducted in a completely randomized design in a factorial $2 \times 2 \times 2$, with four replications for each treatment. Factors were 'mycorrhizal inoculation, M', 'humic substances, HS' and 'CO_2 concentration in the atmosphere, CO_2'.

The following day after transferring seedlings to trays (on 18th April 2013) half of cells (80 seedlings) received 5 mL of a solution containing 20% HS (20HS). This solution was applied by using a syringe. The original commercial solution had 10% FA, 90% HS and pH 4.0, originating from Leonardita (Nutriplant®), 34.4% C; 3.8% H and 2.3% N. Other 80 seedlings did not receive this solution (0HS). Two days after transferring seedlings to trays (on 19th April 2013), half of 0HS cells (40 seedlings) and half of 20HS cells (40 seedlings) were inoculated with the liquid mycorrhizal inoculum 'Glomygel Intensivo' (Mycovitro S.L., Pinos Puente, Granada, Spain) (+M seedlings). The concentrated commercial inoculum derived from an *in vitro* culture of arbuscular mycorrhizal fungi (AMF): *Rhizophagus intraradices* (Schenck and Smith) Walker & Schüβler comb. nov. (Krüger et al., 2012). It contained around 2000 mycorrhizal propagules (inert pieces of roots colonized by AMF, spores and vegetative mycelium) per mL of inoculum. In order to facilitate its application, the concentrated commercial inoculum was diluted with distillate water until obtaining a resultant mycorrhizal inoculum with around 250 propagules per mL. Each +M seedling received 5 mL of the diluted mycorrhizal inoculum close to the roots thus making a total of 1250 propagules. A filtrate was added to seedlings that did not receive the mycorrhizal inoculum (−M seedlings) in an attempt to restore other soil free-living microorganisms accompanying AMF. The filtrate was obtained by passing diluted mycorrhizal inoculum through a layer of 15–20 μm filter papers (Whatman, GE Healthcare, UK) and each −M seedling received 5 mL of filtrate close to the roots. The selection of in vitro-produced inoculum of *R. intraradices* was based on two expected benefits: (1) easy application of the product and (2) low colonization of onion roots by contaminant fungi (Vimard et al., 1999).

Subsequently all trays were transferred to four [CO_2] controlled greenhouses located at the University of Navarra campus (42.80 N, 1.66 W; Pamplona, Spain). The design of the greenhouses was similar to that described by Sanz-Sáez et al. (2012) and based on Aranjuelo et al. (2005). Half of seedlings (20 0HS +M seedlings, 20 0HS −M seedlings, 20 20HS +M seedlings and 20 20HS −M seedlings) were divided into two greenhouses where no CO_2 was added and [CO_2] was maintained at ambient conditions (~360 μmol mol^{-1}) (ACO_2). The other half (20 0HS +M seedlings, 20 0HS −M seedlings, 20 20HS +M seedlings and 20 20HS −M seedlings) were divided into two greenhouses where [CO_2] was increased to ~700 μmol mol^{-1} by injecting pure CO_2 (purity up to 99.99%) from cylinder-gases (34 L of CO_2 per cylinder) at the two inlet fans during the light hours (ECO_2). Injection of CO_2 to greenhouses began when light intensity was equal or superior to 5 W m^{-2} as measured by a Silicon Pyranometer PYR-S (APOGEE Instruments, Inc., Logan, UT, USA) making a total of 13–15 h of high CO_2 a day from April to June. The CO_2 was provided by Air Liquide (Bilbao, Spain). The [CO_2] was continuously monitored using a Guardian Plus gas monitor (Edinburgh Instruments Ltd, Livingston, UK). The monitor's signal was fed into a proportional integrative differential controller that regulated the opening time (within a 10 s cycle) of a solenoid valve that injected CO_2 into both inlet fans.