Phenotyping of *Brassica napus L.* plantlets affected during *in vitro* growth by the presence of epoxiconazole.

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Abstract

Epoxiconazole like others triazole fungicides are known to be persistent in the soil. Several studies using foliar application experiments demonstrated the effect of its triazole metabolite as plant growth regulator through the anti-gibberellin activity. And notably, the reduction of *Brassica napus L.* growth can be attributed to the inhibition of gibberellin biosynthesis at the stage of conversion of *ent*-kaurene to *ent*-kaurenoic acid. We describe here an *in vitro* experiment studying the relationship between epoxiconazole in culture medium (0 mg L^{-1}, 0.120 mg L^{-1} and 0.200 mg L^{-1}) and the phenotyping (root and shoot growth) of three varieties of winter rapeseeds (*Brassica napus L.* var. *Catalina,* var. *ES Astrid* and var. *Toccata*). Plantlets fungicide content was quantified using the QuEChERS extraction method following by an automated UHPLC-MS/MS analysis. Results showed that the shoots and roots growth of *Brassica napus L.* plantlets was significantly inhibited by epoxiconazole at 0.120 mg L^{-1} independently of the variety. The concentration of 0.200 mg L^{-1} leaded to necrosis and anthocyanosis symptoms and can be considered as lethal for *in vitro* growing explants. The huge epoxiconazole absorption by rapeseed plantlets clearly showed a dose-dependent relationship and was closely similar for the three varieties.

Keywords: triazole, oilseed rape, growth regulation, fungicide absorption

INTRODUCTION

Epoxiconazole ((2RS,3SR)-1-[3-(2-chlorophenyl)-2,3-epoxy-2-(4-fluorophenyl)propyl]-1*H*-1,2,4-triazole) is a broad-spectrum triazole fungicide interfering with the biosynthesis of ergosterol, a fungal essential membrane component, by competitively inhibiting the enzyme lanosterol 14α-demethylase (Chambers et al., 2014). Foliar application to the field corresponding to the agronomic homologated dose of 125 g ha^{-1} is mainly used for preventive and curative actions regarding cereals, sugar beets, apple trees, oilseed rape and ornamentals (Liang et al., 2012; Lichiheb et al., 2015). The triazole fungicides exhibit long persistence in soil and especially, epoxiconazole with a half-life time greater than two years at 10°C and 80% of field capacity (Bromilow et al., 1999). Field accumulation studies showed a plateau concentration of 0.167 mg Kg^{-1} into soils (EFSA, 2008).

Triazole-type compounds such as metconazole and tebuconazole are also frequently used on oilseed rape crop for both their plant growth regulatory effect and fungicidal properties (Berry and Spink, 2009). Experiments with foliar application of epoxiconazole at laboratory-scale clearly indicated that phytosterol biosynthesis is affected (Benton and Cobb, 1997) and that epoxiconazole reduces electron transport capability of thylakoids (Petit et al., 2012). A study in hydroponic conditions described a decrease of oilseed rape plant height and shoot biomass related to the use of a triazole type plant growth regulator (Bruns et al., 1990). In fact, triazole compounds show an anti-gibberellin activity by inhibition of the early steps of their biosynthesis at the stage of conversion of *ent*-kaurene to *ent*-kaurenoic acid (Rademacher, 2000; Yamaguchi, 2008). We decided therefore to set up

experiments in order to test for a first time the *in vitro* phenotyping effect of epoxiconazole presence on plantlet growth of three varieties of winter rapeseeds (*Brassica napus L.* var. *Catalina,* var. *ES Astrid* and var. *Toccata*) and to evaluate the epoxiconazole absorption by the rapeseed plantlets.

MATERIALS AND METHODS

Plantlets growth

The three winter oilseed rape varieties of *Brassica napus L.* var. *Catalina* (Dekalb, France), var. *ES Astrid* (Euralis semences, France) and var. *Toccata* (Maïsadour semences, France) germinated *in vitro* from certified seeds and micropropagated by axillary branching. Three explants were cultivated using *in vitro* home-developed container system (weck®). Plantlets (rooting and development of the standardized shoots) were obtained after 36-days of culture without plant growth regulator (medium composition: 400 mg L^{-1} NH_4NO_3; 800 mg L^{-1} KNO_3; 300 mg L^{-1} $Ca(NO_3)_2$; 180 mg L^{-1} $MgSO_4$; 150 mg L^{-1} KH_2PO_4; 1.5 mg L^{-1} $MnSO_4$; 0.5 mg L^{-1} $ZnSO_4$; 3 mg L^{-1} H_3BO_3; 0.5 mg L^{-1} KI; 0.25 mg L^{-1} Na_2MoO_4; 20 mg L^{-1} EDTA*Na_2; 15 mg L^{-1} $FeSO_4$ supplemented with 3% sucrose and 0.5% agar). Plantlets were cultivated under controlled environmental conditions within a climate room at 23/18°C (day/night), using a 16h photoperiod, 40% relative humidity and 10 µmol m^{-2} s^{-1}. In parallel to a control medium, epoxiconazole (Sigma-Aldrich, Diegem, Belgium) was added by filtration using 0.2 µm supor membrane syringe filter (nonpyrogenic AcrodiscR®) after autoclaving at a concentration of 0.120 mg L^{-1} and 0.200 mg L^{-1}. Experiment was performed in triplicate for each variety. Phenotyping corresponded to leaf symptoms observation and to plantlets height and roots length recording.

Epoxiconazole extraction and liquid chromatography analysis

Extraction of epoxiconazole from plantlets was performed using the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method. The three whole plantlets were crushed together with a blender before making 2-g of minced sample weighed into a 50-mL centrifugation tube, homogenized with 5 mL of deionized water and macerated 30 minutes before adding of 10 mL acetonitrile. QuEChERS salts were added to perform phase-separation following by agitation and centrifugation (4500 r min^{-1}). The supernatant was filtered through a 0.20 µm filter (PTFE) into a sample vial for UHPLC-MS/MS analysis. The liquid chromatograph used was a Waters and separation was performed using a C18 column (50 mm x 2.1 mm x 1.7 µm) with mobile phase composed of a mixture of H_2O/methanol/formic acid (90:10:0.1, v/v/v) and methanol/formic acid (100:0.1, v/v) with an elution gradient (80-20%) at 35°C and at flow rate of 0.3 mL min^{-1}. An Acquity® (Waters) triple-quadrupole detector mass spectrometer equipped with an electrospray ionization source was used for MS/MS analysis. The transitions of precursor ion (m/z 330) to production (m/z 121 and 101) of epoxiconazole were detected with multiple reaction monitoring (MRM) in positive ion (ESI+) mode. The collision energies for m/z 121 and 101 were respectively 22 and 50 eV and the ion m/z 121 was used for quantification based on matrix-matched calibration curve obtained from linear regression of plotted area for respective epoxiconazole concentrations gradient (0.001-0.5 µg mL^{-1}).

RESULTS AND DISCUSSION

Phenotyping results

Epoxiconazole presence in the culture medium was clearly responsible of a visual effect on *Brassica napus L.* plantlets growth physiology after 36-day of *in vitro* culture (Figure 1). The rooting of shoots was tremendously inhibited and it can be observed for

0.120 mg L^{-1} and 0.200 mg L^{-1} epoxiconazole concentrations both. The three winter rapeseed varieties (var. *Catalina*, var. *ES Astrid* and var. *Toccata*) were also similarly affected by a reduction of plantlets internode certainly due to the triazole metabolite anti-gibberellin activity (Bruns et al., 1990; Berry and Spink, 2009). Phytotoxic symptoms such as leaf chlorosis and decreasing growth were found at 0.120 mg L^{-1} confirming the photosynthetic apparatus perturbation by epoxiconazole (Petit et al., 2012). Finally 0.200 mg L^{-1} epoxiconazole concentration leaded to severe chlorosis, anthocyanosis mainly for var. *ES Astrid* and could be considered as lethal in regards to harvested plantlets morphology and specially, for the inhibition of root system development.

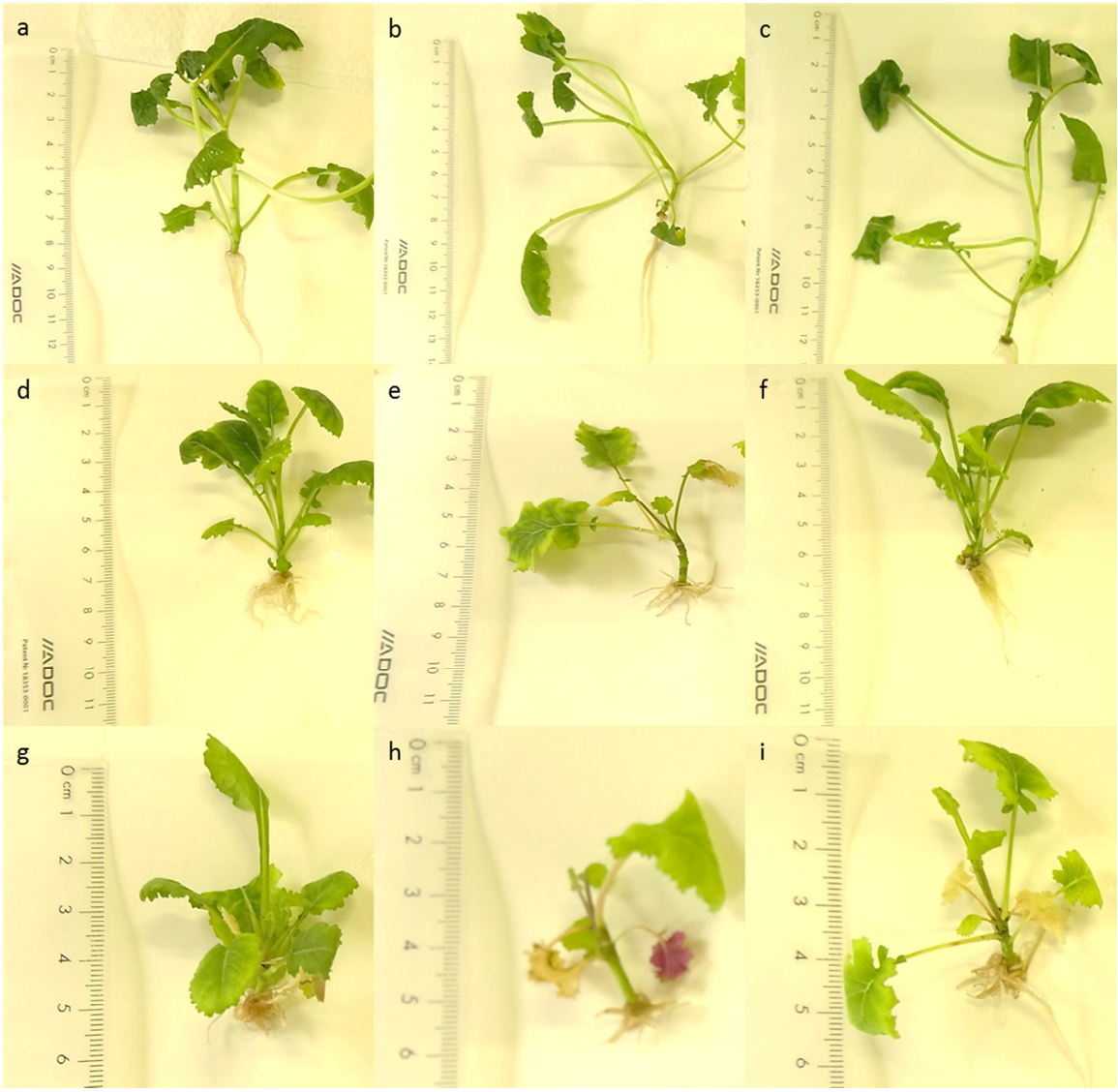

Figure 1. Pictures of *Brassica napus L.* plantlets after 36-day of culture on control medium (a: var. *Catalina*, b: var. *ES Astrid*, c: var. *Toccata*); on medium with 0.120 mg L^{-1} of epoxiconazole (d: var. *Catalina*, e: var. *ES Astrid*, f: var. *Toccata*) and on medium with 0.200 mg L^{-1} of epoxiconazole (g: var. *Catalina*, h: var. *ES Astrid*, i: var. *Toccata*).

Visual interpretation of plantlets observation was corroborated with data obtained from phenotyping results (plantlets height and roots length) concerning the three winter rapeseed varieties (var. *Catalina*, var. *ES Astrid* and var. *Toccata*) (Table 1). A significant

decrease can be observed directly from 0.120 mg L^{-1} of epoxiconazole concentration regarding plantlets height and roots length means both and independently of the variety (Figure 2).

Table 1. Phenotyping results (plantlet height and root length mean ±SE) of *Brassica napus L.* plantlets cultivated under 0, 0.120 mg L^{-1} and 0.200 mg L^{-1} of epoxiconazole (var. *Catalina*, var. *ES Astrid* and var. *Toccata*).

Epoxiconazole Concentration	**var. *Catalina***	**var. *ES Astrid***	**var. *Toccata***
Plantlet height mean[a] (mm)			
0 mg L^{-1}	117±2	123±4	128±7
0.120 mg L^{-1}	75±6	65±6	63±6
0.200 mg L^{-1}	52±3	46±3	51±4
Root length mean[a] (mm)			
0 mg L^{-1}	116±15	159±11	71±8
0.120 mg L^{-1}	30±2	21±4	28±2
0.200 mg L^{-1}	17±2	6±1	15±3

[a] : n=9

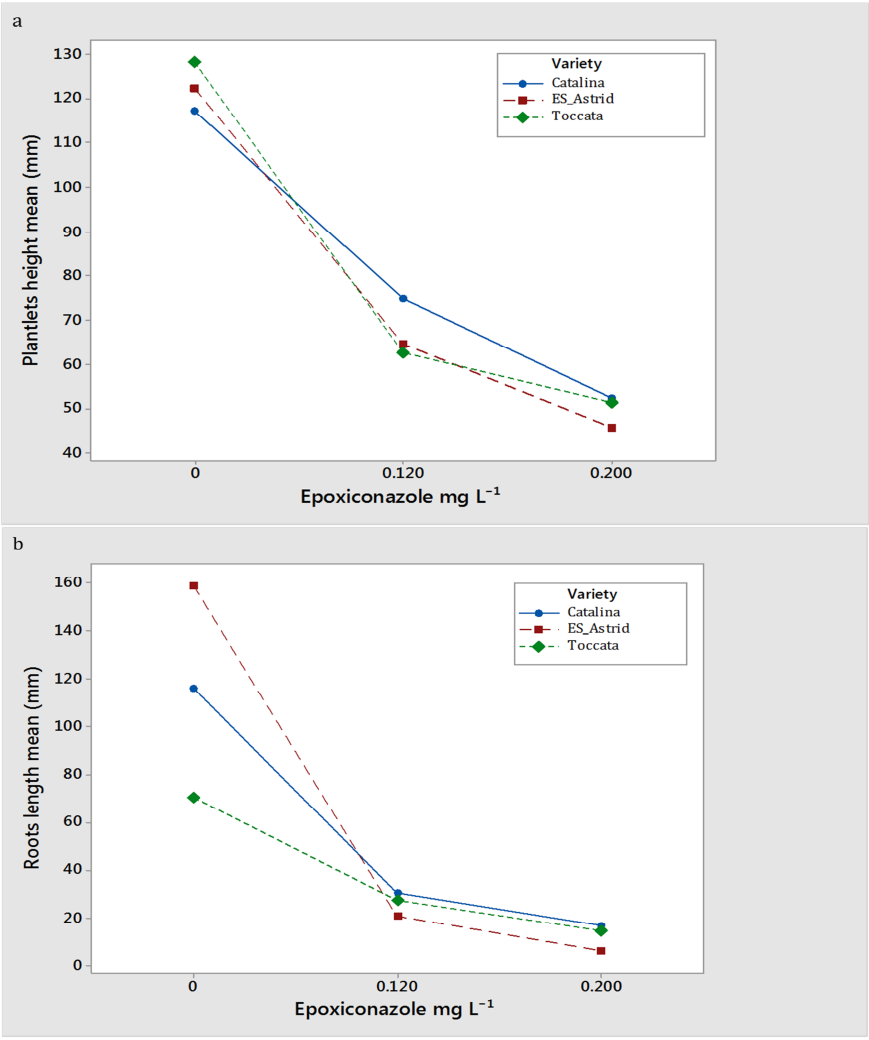

Figure 2. Graphs showing the phenotyping results for *Brassica napus L.* (var. *Catalina*, var. *ES Astrid* and var. *Toccata*) plantlets cultivated under 0, 0.120 mg L^{-1} and 0.200 mg L^{-1} of epoxiconazole a) plantlet height means (n=9); b) root length means (n=9).

Epoxiconazole absorption

UHPLC-MS/MS analysis of epoxiconazole content of the *Brassica napus L.* plantlets showed an obvious dose-dependent relation between its level in the culture medium and its absorption (Figure 3). Means (±SE) of epoxiconazole absorption (µg g^{-1}) were closely similar for the three varieties respectively (var. *Catalina*, var. *ES Astrid* and var. *Toccata*): 0.59(±0.08), 0.57(±0.03), 0.61(±0.01) for 0.120 mg L^{-1}; 1.30(±0.05), 1.6(±0.07), 1.6(±0.17) for 0.200 mg L^{-1}. It could be concluded in comparison with phenotyping results that epoxiconazole plantlets absorption lead to severe stress physiology from 0.6 µg g^{-1}. Liquid chromatography analytics research and development could help to evaluate in the future the epoxiconazole metabolization rate into triazole compounds for a better understanding of the morphology of the observed symptoms.

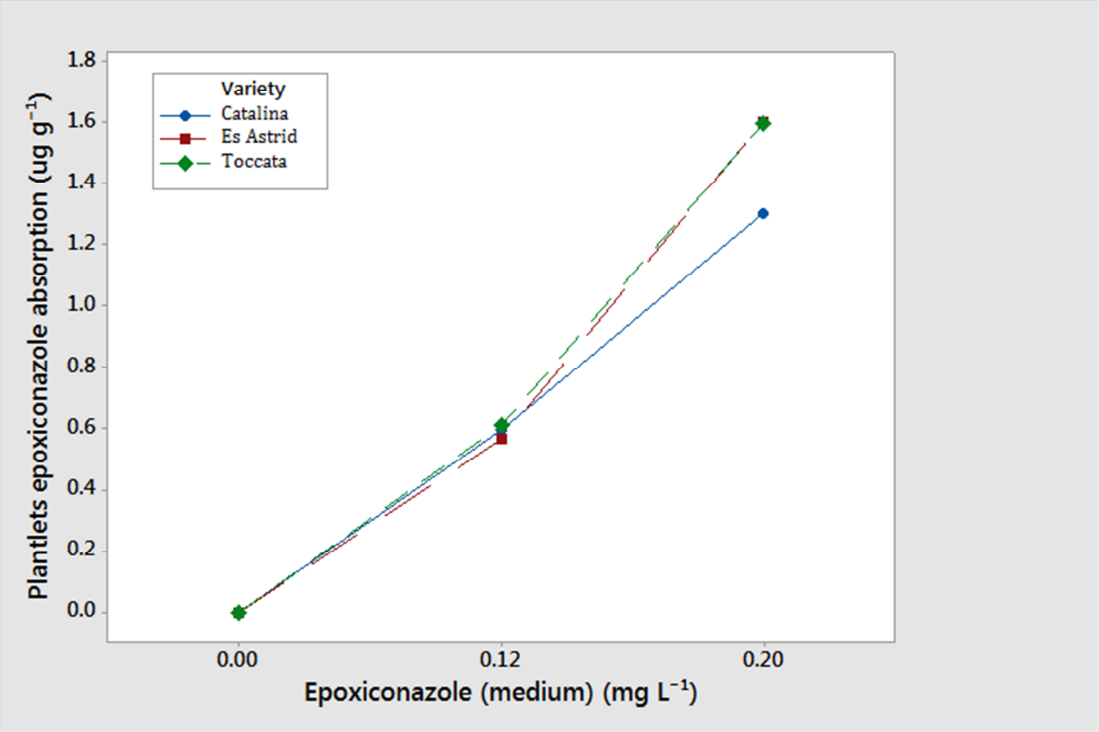

Figure 3. Graph of epoxiconazole absorption mean (n=3) by the plantlets of *Brassica napus L.* (var. *Catalina*, var. *ES Astrid* and var. *Toccata*) cultivated under 0, 0.120 mg L^{-1} and 0.200 mg L^{-1} of epoxiconazole.

CONCLUSIONS

The experiments performed on *in vitro* growing *Brassica napus L.* (var. *Catalina*, var. *ES Astrid* and var. *Toccata*) explants lead to the following conclusions:

- Confirmation that the presence of epoxiconazole affected clearly the plantlets growing physiology within sterile and controlled conditions.
- Decreasing growth (plantlet height), inhibition of root system development and severe chlorosis were observed at 0.120 mg L^{-1} of epoxiconazole and epoxiconazole at 0.200 mg L^{-1} can be considered to be a lethal concentration.

It will be interesting to confirm these observations with future experiments using oilseed rape cultivated on artificially treated substrate to evaluate putative phytotoxicity related to triazole fungicides persistence (Bromilow et al., 1999; Liang et al., 2012) and in the frame of multiple abiotic stress conditions.

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