Phytotoxic and metabolic effects of exogenous quinate on Pisum sativum L.

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Keywords:	Spray to leaves, supply to roots, phytotoxicity, physiological effects, glyphosate, acetolactate synthase inhibitor

Phytotoxic and metabolic effects of exogenous quinate on *Pisum sativum* L.

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Shortened version of the title: Phytotoxic effects of exogenous quinate

Abstract Quinate (1,3,4,5-tetrahydroxycyclohexanecarboxylate) is a compound synthesized in plants through a side branch of the shikimate pathway. Plants treated with herbicides that inhibit amino acid biosynthesis (branched-chain and aromatic) accumulate quinate in their leaves. The objective of this study was to evaluate whether quinate mimics the effects of herbicides in plants. In pea plants, exogenous application of quinate through the nutrient solution was compared with leaf spraying at a concentration of 4 mM and 400mM, respectively, and evaluated in parallel to the effects of herbicides. The analysis facilitated an assessment of the phytotoxicity and potential use of quinate as a natural herbicide. The application of quinate through the nutrient solution, but not the spray, was lethal, although both treatments affected plant growth. Quinate was absorbed and translocated to other plant organs remote from the application site, and an increase in the levels of aromatic amino acids and caffeic acid (i.e., compounds located after quinate in the shikimate biosynthetic pathway) was detected, which indicates that quinate was metabolized and incorporated into the shikimate pathway. Exogenous application of quinate affected the carbohydrate content in the leaves and roots similarly to the toxic effects of herbicides. The phytotoxic effects of quinate reported in this study suggest that this compound deregulates the shimikate pathway and mimics some physiological effects described in the mode of action of herbicides inhibiting amino acid biosynthesis.

Key Words spray to leaves, supply to roots, phytotoxicity, physiological effects, glyphosate, acetolactate synthase inhibitors.

Introduction

Currently, more than 60 structurally different active herbicide ingredients are in use. Imidazolinones are one of the five herbicide classes that inhibit acetolactate synthase (ALS, also known as acetohydroxyacid synthase; EC 4.1.3.18) as their primary target (Ray, 1982; Shaner and others 1984).

These inhibitors have become one of the most important herbicide groups because of their wide-spectrum weed control activity, high crop selectivity, low application rate and low mammalian toxicity (Zhou and others 2007). ALS is the first common enzyme in the biosynthesis of the branched-chain amino acids valine, leucine and isoleucine.

Glyphosate (N-(phosphonomethyl) glycine) is a wide-spectrum, non-selective post-emergence herbicide, and since its commercial introduction in 1974, glyphosate has become the predominant and most popular herbicide used worldwide (Duke and Powles, 2008). Glyphosate inhibits the biosynthesis of the aromatic amino acids tyrosine, phenylalanine and tryptophan in chloroplasts via the shikimate pathway. Glyphosate specifically inhibits the enzymatic activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) (Steinrücken and Amrhein, 1980).

Although the primary effects of these two types of herbicides are widely known, the mechanisms underlying plant death after the inhibition of ALS or EPSPS remain unclear. EPSPS and ALS inhibitors both arrest the growth of treated plants, and this growth arrest is followed by a slow death (Wittenbach and Abell, 1999; Gruys and Sikorski, 1999). Several physiological effects common to both glyphosate and ALS inhibitors have been reported (Orcaray and others 2010), including a general increase in the total free amino acid content with a transient decrease in the proportion of amino acids whose pathways are specifically inhibited. A second common effect is the accumulation of quinate in the leaves of plants treated with both types of herbicides (Orcaray and others 2010). Quinate (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid) is a metabolite synthesized in a lateral branch of the shikimate biosynthetic pathway in plants.

Plants possess two mechanisms for the synthesis of quinate: dehydroquinate catalyzed by quinate dehydrogenase and shikimate catalyzed by quinate hydrolyase (Bentley, 1990; Leuschner and others 1995). Quinate occurs in relatively high concentrations in green and non-green tissues of herbaceous plants (Yoshida and others 1975) and in young, developing tissues of conifers (Osipov and Aleksandrova, 1986) and fruits (Albertini and others 2006). Quinate is considered a reserve compound of the shikimate pathway, although its physiological role has not been completely clarified.

Currently, researchers are developing new pesticides to replace compounds that no longer meet environmental or toxicological safety requirements. However, the battle against the evolution of weed resistance requires the discovery and development of new herbicides that inhibit different biochemical targets to alleviate selection pressure caused by currently used herbicides (Dayan and others 2009; Duke, 2012).

The development of new toxicologically and environmentally safe products to replace compounds banned through legislation is also desirable. Natural-product-based herbicides have been proposed as alternatives to conventional herbicides. These compounds are considered to be safer than synthetic herbicides because of their often relatively short life, which is desirable from an environmental point of view. Nevertheless, good herbicides must persist long enough to be effective. The short life of natural herbicides and other disadvantages, such as structural complexity, have limited the efforts of the herbicide industry in natural product discovery (Dayan and others 2012).

ALS and EPSPS inhibitors both induce quinate accumulation in plant leaves. This physiological effect has been implicated in the toxicity of these herbicides, which begs the question of whether quinate mediates the toxic effects or mimics the action of the herbicides. In the latter case, exogenous application of this compound could potentially be used as a commercial herbicide treatment. However, elucidation of the mode of action is necessary for the potential use of the natural phytotoxins as novel tools for weed management (Dayan and others 2000).

To evaluate the potential phytotoxic effects of quinate, commercially available quinate was applied to the nutrient solution or leaves of pea plants and the effects of these treatments were discussed in comparison with the effects of amino acid biosynthesis inhibitors. The objective of this study was to determine whether this compound mimics the action of herbicides and causes sufficient toxic effects on plants to be used as a herbicide.

Materials and methods

Plant Material and Treatment Application

Pisum sativum L. cv. Snap Sugar Boys was grown in aerated hydroponic culture as described in Zabalza and others (2005). At 12 days of age, the plants were divided into two groups, one to assess the nutrient solution treatments, and the other one to assess the spray treatments to the leaves. Plants were treated with quinate (Quinic acid 98%, Sigma, St. Louis, MO, USA). In the first group, half of the plants were treated with 4 mM quinate to the nutrient solution (quinate treatment) and the other half was not treated and served as the control treatment. The other group was subsequently used to assess the application of quinate to the leaves. The experiment was repeated twice.

Quinate was sprayed onto the leaves of the half of the plants using a mechanical sprayer at a concentration of 400 mM in 5.4% of the adjuvant sodium lauryl sulfate (commercial formula Biopower 27.65% (p/v), Bayer CropScience, Madrid, SPAIN). Control plants were sprayed with adjuvant only. After monitoring quinate concentration in the nutrient solution during experiments, it was established that refreshing the nutrient solution every 3 days was enough to minimize microbial contamination and to maintain quinate availability. So, the nutrient solution was replaced every 3 days.

Plant samples were obtained at 0, 1, 3, 7, 10 and 15 days post treatment, and in some cases (indicated), the study only included sampling on days 7 or 15. At harvest, the samples were collected, immediately frozen in liquid nitrogen and stored at -80 ºC for analytical determinations. For the sprayed plants, the samples were classified as leaf material present at the time of quinate application and leaves appearing after quinate treatment. The leaves present at the time of quinate application were washed to remove quinate from the surface before freezing.

Analytical Determinations

Quinate content in leaves and roots of pea plants was extracted in TCA and measured using ion chromatography as previously described (Orcaray and others 2010). The caffeic, ferulic and *p*-coumaric acid contents were assessed using HPLC, as previously described for hydroxybenzoic acid determination (Orcaray and others 2010). The extraction of amino acids was done in HCl. After protein precipitation, amino acid concentrations were measured in the supernatant using capillary electrophoresis equipped with a laser-induced fluorescence detector, as previously described (Orcaray and others 2010). For the carbohydrate determination, leaves and roots were extracted in ethanol 80%, the ethanol-soluble extracts were dried in a Turbovap (Zymark, Hopkinton, MA), and the soluble compounds were redissolved in distilled water. The ethanol-insoluble residue was extracted for starch. Then, the content of glucose, sucrose and starch was determined using capillary electrophoresis as previously described (Zabalza and others 2004).

Statistical Analysis

Unpaired Student's t-test was used to determine the significance of differences. Each mean value was calculated using samples from single plants as biological replicates coming from two different experiments. Significant differences ($p < 0.05$) between each treatment and each control plant (untreated plants) are indicated in the figures with different symbols on the given day of treatment. When the values analyzed were percentages, a previous transformation to arcsine $\sqrt{(x/100)}$ was used.

Results

Effects of Quinate Treatment on Growth

Quinate was applied through the nutrient solution and sprayed onto the leaves of plants, and the effects on growth, quinate and hydroxycinnamic acid content, amino acid pattern and carbohydrate content were measured for both treatments.

Preliminary studies were conducted to investigate the lethality and effects on growth of different concentrations of quinate delivered through the nutrient solution. The response of pea plants was dose dependent. While 1 mM was sublethal, 10 mM and 50 mM of quinate caused plant death after 10 and 3 days of treatment respectively. Finally, the study was carried out with 4 mM, because this dose caused plant death after 3 weeks, similarly to the lethality reported following the concentrations of ALS and EPSPS inhibitors whose effects were used for the comparison study. The selection of 4 mM quinate to be applied to the nutrient solution was validated by confirming that quinate accumulation in leaves after its exogenous supply was similar to the levels detected after ALS or EPSPS inhibition by herbicides (Orcaray and others 2010). This treatment to the nutrient solution was compared with a concentration of 400 mM quinate applied to the leaves, as this one was established as the maximum solubility of quinate in the spray mix [the adjuvant dissolved in water] without any subsequent precipitation on the leaf surface.

Both treatments arrested shoot elongation. The growth inhibition was more rapid when quinate was sprayed onto the leaves than when it was applied to the nutrient solution (within the seventh and tenth days, respectively) (Fig. 1a, b). In both cases, shoot growth inhibition rate was approximately 60% after 15 days of treatment. In contrast, root growth was only affected when quinate was applied to the nutrient solution (Fig. 1c, d), in which case inhibition was complete.

Whereas 4 mM quinate by nutrient solution treatment caused plant death after 3 weeks, 400 mM quinate by foliar spray was sublethal but induced necrosis in the terminal bud of the shoot apex of the sprayed plants (data not shown).

Effects of Exogenous Application of Quinate on Quinate and Hydroxycinnamic Acid Content

Application of quinate to the leaves or roots dramatically increased the concentration of quinate in the leaves and roots (Fig. 2), which shows that quinate was absorbed and translocated to plant organs remote from the application site. The quinate concentration detected in the leaves was higher in spray-treated plants than in plants treated through the nutrient solution.

In the sprayed plants, the quinate content was measured separately in the leaves present during the application of quinate (referred to as “old” leaves) and in the leaves appearing after quinate application (referred to as “new” leaves). Whereas quinate accumulation was significant in old leaves throughout the treatment, the quinate content peaked in new leaves after 3 days and disappeared after 10 days (Fig. 2b).

The accumulation of quinate in the roots was more evident when quinate was supplied through the nutrient solution than when it was sprayed onto the leaves (Fig. 2c and d). In quinate-sprayed plants, the accumulation of quinate in roots peaked at day 3 and subsequently diminished, similarly to the pattern detected in new leaves (Fig. 2d).

The content of three hydroxycinnamic acids (caffeic, ferulic and *p*-coumaric acids) was measured in the leaves after both types of treatment (Fig. 3). Only the leaves of plants supplied with quinate through the nutrient solution showed effects in the content of caffeic, ferulic and *p*-coumaric acids. However, different accumulation patterns were observed for each acid. The p-coumaric acid content was significantly reduced at days 7 and 15 from the onset of treatment (Fig. 3e), whereas the content of caffeic and ferulic acid was significantly increased (Fig. 3a, c).

Effects of Exogenous Application of Quinate on Free Amino Acid Content

Figure 4 shows the total free amino acid content, the proportion of aromatic amino acids (sum of phenylalanine, tyrosine and tryptophan in relation to total free amino acids) and the proportion of branched-chain amino acids (sum of valine, leucine and isoleucine in relation to total free amino acids) in the leaves.

When quinate was applied through the nutrient solution, the total free amino acid content and the percentage of branched chain amino acids were reduced relative to the values in the control plants (Fig. 4a, e). In contrast, the percentage of aromatic amino acids increased within 7 days to levels higher than the control values (Fig. 4c).

Spraying quinate onto the leaves increased the short-term free amino acid pool, and this increase was detected in both old and new leaves (Fig. 4b). In old leaves, the percentage of branched and aromatic amino acids was higher in quinate-treated plants, whereas no differences were detected in the new leaves (Fig. 4d, e).

Carbohydrate Content in Leaves and Roots after Exogenous Application of Quinate

Application of quinate through the nutrient solution induced carbohydrate accumulation as both soluble carbohydrates (glucose and sucrose) and starch in the leaves. The glucose content under quinate treatment was 10 times higher than that in non-treated leaves on day 15. Sucrose and starch accumulation were significant within 7 days from the onset of the treatment (Fig. 5). Similarly, the carbohydrate content in the roots after quinate was added to the nutrient solution was higher than that in the control roots. Non-significant accumulation of sucrose and starch was observed at the end of the study period at days 7 and 10 (Fig. 6).

The leaf carbohydrate content was not affected after quinate was sprayed onto the foliage (Fig. 5). Notably, in the leaves of sprayed plants, quinate treatment did not affect the starch content; however, the starch content was affected by the age of the tissue, as it increased at the end of the study period in leaves that appeared after the spray treatment (Fig. 5f). Consistent with the carbohydrate content in the leaves, the content of sucrose and starch was not significantly changed in the roots of pea plants sprayed with quinate (Fig. 6b, d).

Discussion

Secondary plant metabolites, which are also known as natural products, are considered to be "a vast repository of materials and compounds with evolved biological activity, including phytotoxocity", and some of these compounds may be useful as herbicides or templates for herbicide development (Duke and others 2002).

Quinate is a compound synthesized in a lateral branch of the shikimate pathway. Although its physiological role has not been completely elucidated, quinate accumulation has been detected in plants during fungal invasion (Parker and others 2009) and after treatment with herbicides that inhibit amino acid biosynthesis (Orcaray and others 2010). This accumulation indicates the potential phytotoxic effect of the exogenous application of quinate and suggests that it could be potentially used for the development of herbicides based on natural products.

In this study, we determined the physiological processes that were affected by quinate, because understanding the plant response mechanism will provide information concerning potential applications of quinate. In particular, we compared the physiological effects observed after two different methods of quinate application, i.e., through the nutrient solution and through spraying onto the foliage.

The two types of exogenous quinate application similarly inhibited pea shoot growth, and shoot elongation was arrested after 7 days (Fig. 1a, b). Root elongation was only arrested when quinate was supplied through the nutrient solution (Fig. 1c, d). Inhibitory effects on plant growth of other metabolites associated with the shikimate pathway, as some hydroxybenzoic and hydroxycinnamic acids, have been previously reported (Vaughan and Ord 1990; Macías and others 2007).

Although quinate was not detectable in the roots and was present at low levels in the leaves of control plants, the concentration of quinate significantly increased after exogenous application (Fig. 4). When quinate was applied to the roots, it predominantly accumulated in the leaves, and when sprayed onto the foliage, quinate significantly accumulated in the roots and new leaves, which indicates that quinate is translocated. The quinate content in the old and young leaves of pea plants sprayed with quinate was reduced after 7 days and reached control values by the end of the study period. To explain this result, it is necessary to consider that there was an exogenous application of quinate only at one moment of the plant development. The highest quinate accumulation was detected after the spray. Afterwards, as pea plants continue growing the content of quinate per gram decreased. In contrast, in pea plants supplied with quinate in the nutrient solution, quinate accumulation was maintained over the duration of the experimental period, which is explained by the continuous supply of quinate through absorption by roots.

The most significant physiological effects previously observed for ALS- or EPSPS-inhibiting herbicides, i.e., effects on hydroxycinnamic acid and carbohydrate contents and amino acid patterns, were assessed in the present study in plants supplied with quinate.

The content of caffeic and ferulic acid (Fig. 3a, c) and the percentage of aromatic amino acids (Fig. 4c) in the leaves of plants supplied with quinate in the nutrient solution were significantly increased relative to those in the control leaves at days 7 and 15 from the onset of the treatment. This accumulation of aromatic amino acids and secondary metabolites suggests a coordinated response of the shikimate pathway. Moreover, these data support the idea that plants can use quinate as a carbon source for the biosynthesis of aromatic amino acids (Leuschner and Schultz, 1991a, b). Quinate hydrolyase, which directly converts quinate into shikimic acid in pea roots, links the quinate pool to the shikimate pathway (Leuschner and others 1995).

Although no changes in carbohydrate content in the leaves or roots were detected after quinate was sprayed onto the foliage, quinate supplementation in the nutrient solution induced the accumulation of soluble carbohydrates in the leaves (Fig. 5a, c, e), which might be associated with a lack of translocation of photoassimilates. Despite the carbohydrate accumulation in the leaves, there was no carbohydrate shortage in the roots. Indeed, carbohydrates also accumulated in the roots of quinate-treated plants (Fig. 6a, c). The examination of the carbohydrate content pattern facilitated the evaluation of effects on phloem transport. The increase in the sucrose and starch content in the sinks suggests that sucrose is transported from the leaves to the roots at a higher rate than the metabolic rate in the sinks. Under these conditions, the sugar gradient required for long-distance transport is abolished, and phloem transport is inhibited as a consequence of sink strength. A similar physiological effect has been reported after treatment with ALS- and EPSPS-inhibiting herbicides (Zabalza and others 2004; Orcaray and others 2012).

Exogenous application of quinate to the nutrient solution induced the internal accumulation of quinate, growth arrest, and accumulation of carbohydrates and hydroxycinnamic acid. These results are consistent with physiological effects that have been detected after treatment with ALS or EPSPS inhibitors (Zabalza and others 2004; Orcaray and others 2010; 2011; 2012). Quinate may not have a target site by itself, but when applied exogenously, it would enter in the shikimate pathway and deregulate different processes related with this pathway and therefore would mimic some of the physiological effects shown in the mode of action of herbicides inhibiting amino acid biosynthesis. Taken together, these results indicate that quinate plays an important role in the mode of action of inhibitors of amino acid biosynthesis. As quinate accumulation has been detected after the application of different types of herbicides, this effect can be considered as a physiological marker of herbicidal activity. Such markers can help in the search for new herbicidal active ingredients that are based on natural products to decrease the use of synthetic compounds. Thus, the use of physiological markers to evaluate the potential herbicidal activity of natural compounds can be very useful.

When quinate was applied through the nutrient solution, growth retardation was followed by the necrosis and death of the growing terminal, and eventual plant death occurred after 3 weeks. Moreover, several physiological effects similar to those of two different synthetic herbicides were detected. When quinate was sprayed onto the foliage, only shoot growth was arrested, and the treatment was not lethal. A comparison of the quinate content of plants treated with both types of applications (Fig. 2) revealed that quinate phytotoxicity only occurs when high and lasting quinate accumulation in the tissues is achieved.

The phytotoxic effects of quinate reported in this study suggest that altering quinate levels can be useful in the development of alternative herbicides based on natural products. Although no new target site has been reported after quinate treatment, the results of this study suggest that deregulating plant metabolism by altering the carbon flow through the shikimate pathway can be deleterious to the plant. Nevertheless, the results showed that quinate sprayed onto the foliage was not as phytotoxic as quinate absorbed through the roots and further studies are necessary to evaluate the use of quinate as an active foliar herbicide. The half-life of quinate in soil could limit is applicability. Soil microorganisms and non-enzymatic processes degrade metabolites. The relatively short environmental half-life of natural products is desirable from an environmental toxicology standpoint, but an herbicide must sufficiently persist to achieve the desired effect (Duke and others 2002; Dayan and others 2012).

In the present study, we focused on physiological processes affected by two different methods for exogenous application of quinate. The physiological characteristics of plants exposed to quinate have provided insight into its mode of action. The phytotoxic effects of exogenous application were more evident when quinate was supplied in the nutrient solution than when it was sprayed onto the foliage. Results evidence that quinate accumulation plays an important role in the toxicity induced by inhibitors of amino acid biosynthesis and suggest that in the development of new herbicides it would be helpful to achieve increased quinate levels in their physiological effects.

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Figure legends

Fig. 1 Shoot (A, B) and root length (C, D) of control pea plants or plants treated with quinate supplied through the nutrient solution (A, C) or sprayed onto the leaves (B, D). Mean ± SE (n=8). The symbols indicate significant differences between control and plants treated with quinate through the nutrient solution (*) or on the leaves (#) on a given day ($p \leq 0.05$).

Fig. 2 Quinate content in the leaves (A, B) and roots (C, D) of control pea plants or plants treated with quinate supplied through the nutrient solution (A, C) or onto the leaves (B, D). Mean ± SE (n=4). * indicates significant differences between control and plants treated with quinate through the nutrient solution. # and ^ indicate significant differences between control and quinate-sprayed plants in the leaves present at the time of treatment (washed before determination) and in leaves that appeared after the treatment (new leaves) on a given day ($p < 0.05$), respectively.

Fig. 3 Content of caffeic (A, B), ferulic (C, D), and coumaric (E, F) acid in the leaves of control pea plants or plants treated with quinate applied through the nutrient solution (A, C, E) or sprayed onto the leaves (B, D, F). Mean ± SE (n=3). * indicates significant differences between the control and plants treated with quinate through the nutrient solution. # indicates significant differences between the control and plants treated with quinate through the leaves of spray-treated plants on a given day ($p < 0.05$).

Fig. 4 Total free amino acid content (A, B) and percentage of amino acids, with respect to the total free amino acid content in the leaves of control pea plants or plants treated with quinate applied through the nutrient solution or sprayed onto the leaves. C, D:

aromatic amino acids; E, F: branched-chain amino acids. Mean ± SE (n=3). *, # and ^ are indicated as in Fig. 2.

Fig. 5 Carbohydrate content in the leaves of control pea plants or plants treated with quinate supplied through the nutrient solution (A, C, E) or onto the leaves (B, D, F). A, B: glucose content; C, D: sucrose content; E, F: starch content. Mean ± SE (n=3). *, # and ^ are indicated as in Fig. 2.

Fig. 6 Sucrose (A) and starch (B) content in the roots of control pea plants or plants treated with quinate supplied through the nutrient solution. Mean ± SE (n=3). The symbols indicate significant differences between the control and plants treated with quinate through the nutrient solution (*) or sprayed onto the leaves (#) on a given day ($p \leq 0.05$).

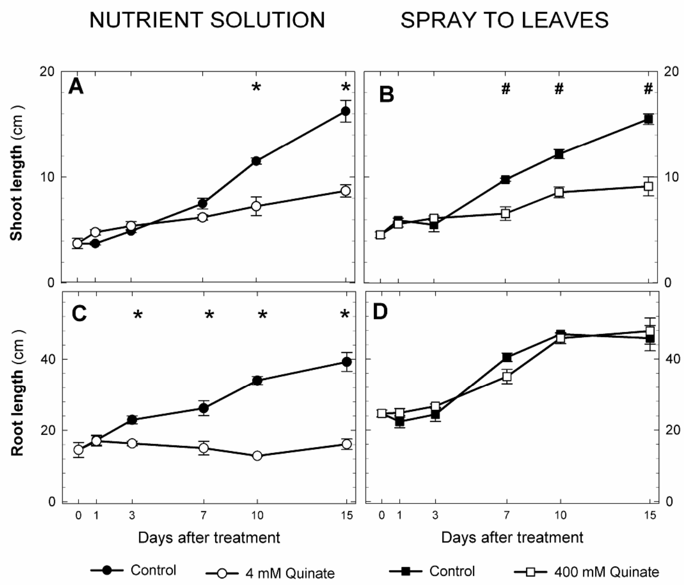

Figure 1
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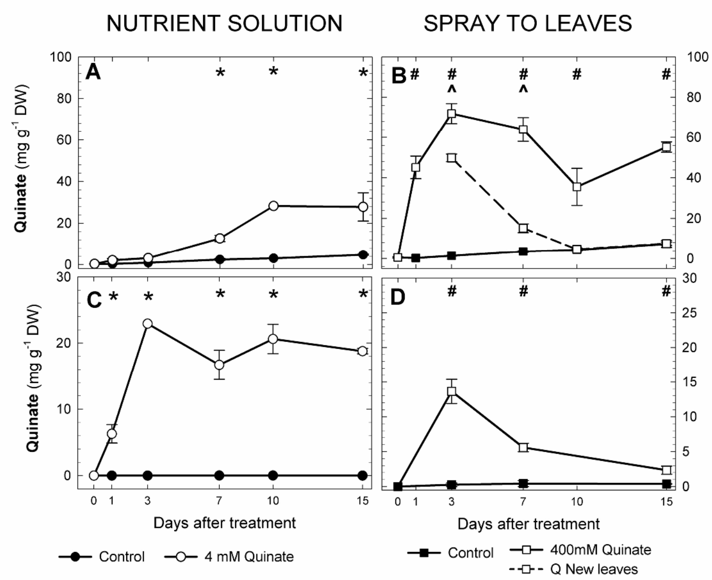

Figure 2
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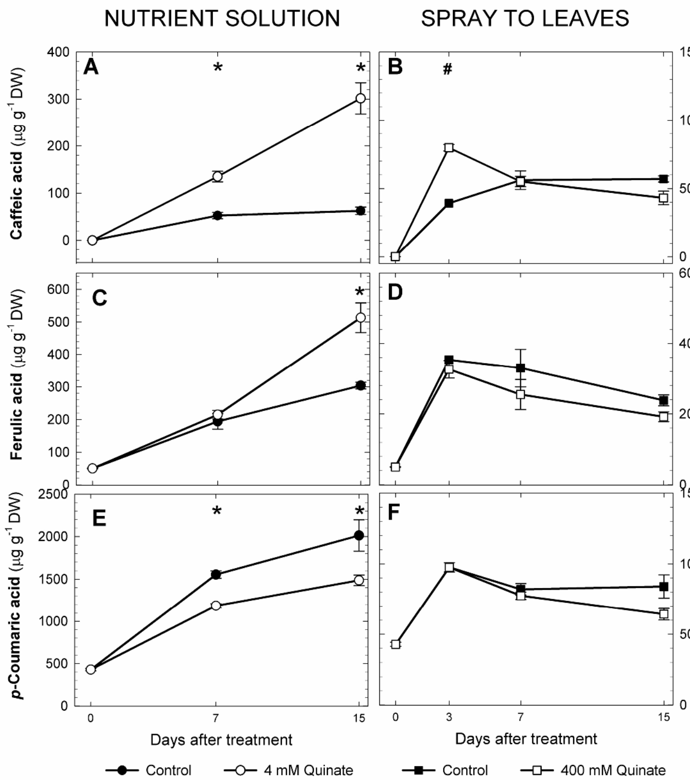

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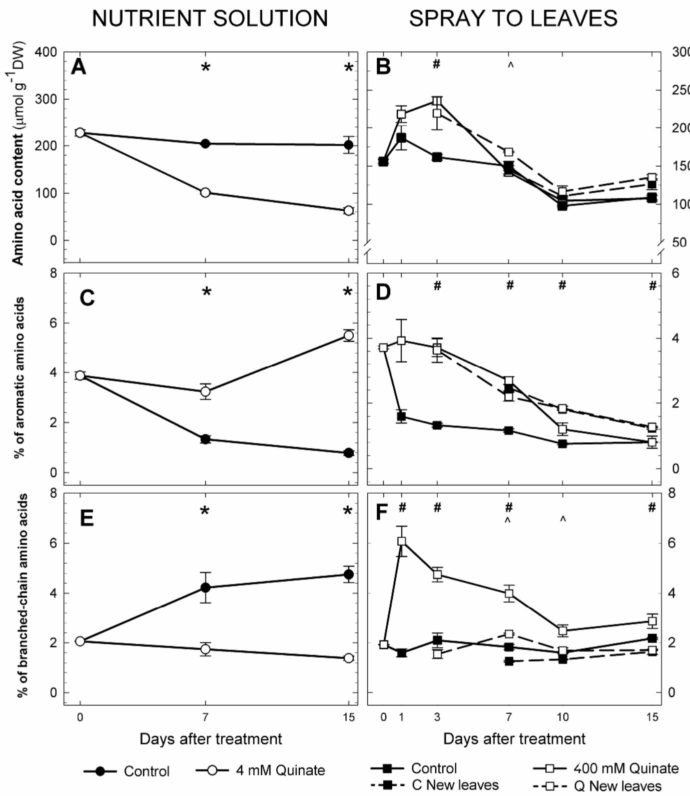

Figure 4
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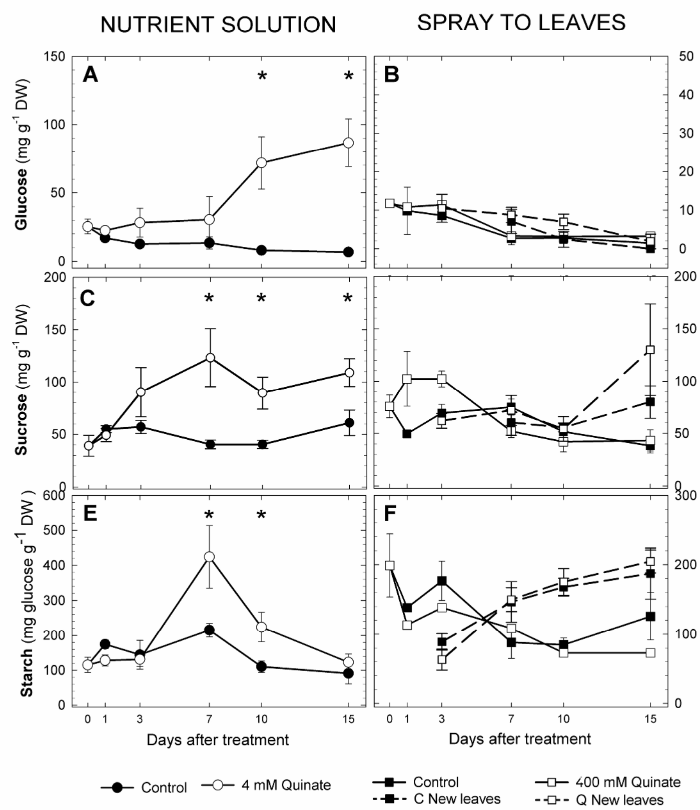

Figure 5
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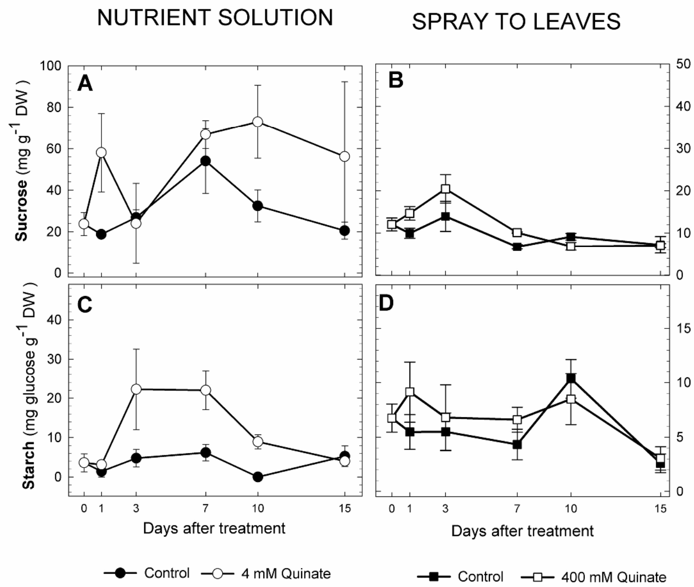

Figure 6
Zulet et al.

297x421mm (300 x 300 DPI)