This is a “preproof” accepted article for Weed Science. This version may be subject to change in the production process, *and does not include access to supplementary material*.
DOI: 10.1017/wsc.2024.75

Primary metabolic profiling of four broomrapes belonging to *Orobanche* and *Phelipanche* species

Evgenia Dor[1#], Aviv Guy[2,3#], Rachel Amir*[2,3], Yael Hacham[2,3]

[1] Newe Ya’ar Research Center, Agricultural Research Organization, P.O. Box 1021, Ramat Yishay 3009503, Israel

[2] Laboratory of Plant Science, MIGAL – Galilee Technology Center, Kiryat Shmona 11016, Israel

[3] Tel-Hai College, Upper Galilee 11016, Israel

[#] Equal contributions

* Corresponding author: Rachel Amir, Laboratory of Plant Science, MIGAL – Galilee Technology Center, Kiryat Shmona 11016, Israel. Tel.: 972-46953516; Fax: 972-6944980; email: rachel@migal.org.il.

Abstract

Genera of the *Orobanchaceae* family are holoparasites that parasitize various hosts. Several members of this family cause severe damage to diverse crop plants. While the biological and life cycles of these parasites have been studied, their metabolism has received little attention, most of which focused on *Phelipanche aegyptiaca*. This study aimed at obtaining more knowledge about the primary metabolic profiling of four parasite species belonging to the *Orobanchaceae* family – *Orobanche cumana*, *Orobanche cernua*, *Phelipanche aegyptiaca* and *Phelipanche ramosa* – that developed on tomato (*Solanum lycopersicum* L.) as a host. Using GC-MS, it was shown that significant differences in metabolites content occur between species belonging to *Orobanche* compared to those belonging to *Phelipanche*. This finding adds another layer to the separation of these two genera in addition to morphological separation. Moreover, each of these four species exhibits different metabolic profiles, indicating that the parasites can absorb the host's metabolites but also have the ability to self-regulate their metabolites in order to grow and develop.

Keywords: GC-MS analysis, holoparasitic plant, *Orobanche*, *Phelipanche*, primary metabolic profiling

Introduction

The genera of broomrape (*Orobanche* spp. and *Phelipanche* spp.) belonging to the *Orobanchaceae* family are obligatory non-photosynthetic root parasites (holoparasitic plants) that can infect a wide range of dicotyledonous host plants (Fernández-Aparicio et al. 2016; Joel 2007). These two genera were previously defined as one genus, *Orobanche*, but based on relatively recent taxonomic characteristics, it was decided to split the broomrape into two separate genera, *Orobanche* and *Phelipanche* (Delavault 2015; Joel 2007; Piwowarczyk et al. 2018). The initial evidence that led to this insight in separating the two species came after examining their phenotypes and morphological characteristics. The flowering stem of *Phelipanche* is branched, while that of *Orobanche* is unbranched (Joel 2009). Furthermore, findings from cytological data, DNA sequences, phylogenetic data, and molecular markers supported the separation (Delavault 2015; Piwowarczyk et al. 2018). Among these findings is that *Phelipanche* has 12 chromosomes while *Orobanche* has 19 (Delavault 2015).

Several species belonging to these genera have invaded agricultural fields over the years and have become one of the most serious pests. The parasite decreases crop quality and causes severe yield reduction in infested fields, reaching up to 100% loss (Fernández-Aparicio et al. 2016; Mutuku et al. 2021). The yield loss occurs mainly in fields infected with sunflower broomrape (*Orobanche cumana* Wallr.), crenate broomrape (*Orobanche crenata* Forssk.), Egyptian broomrape [*Orobanche aegyptiaca* Pers.; syn.; *Phelipanche aegyptiaca* (Pers.) Pomel], and branched broomrape (Orobanche ramosa L.; syn. *Phelipanche ramosa* L.). These parasites parasitize critical crops, thus negatively affecting human nutrition and leading to heavy economic losses (Westwood, 2013). *P. aegyptiaca* and *P. ramosa* have a wide range of hosts, including *Solanaceae*, legumes, cucurbits, and *Apiaceae* (Mutuku et al. 2021). *Orobanche cernua* commonly infests *Solanaceae* (tomato (*Solanum lycopersicum* L.), tobacco (*Nicotiana tabacum* L.), eggplant (*Solanum melongena* L.), and potato (*Solanum tuberosum* L.), while *O. cumana* mainly infects sunflower (*Helianthus annuus* L.) (Cochavi et al. 2017). The ability to infect multiple hosts might offer an ecological benefit, as diverse hosts are likely to provide the parasite with various nutrient types and quantities (Kumar et al., 2022). Genetics and tissue incompatibility govern the number of acceptable hosts for each parasite. However, in practice, the host range is mainly affected by geographical (host distribution) and ecological (dispersal biology and environmental factors) relationships (Casadesús and Munné-Bosch, 2021; Westwood, 2013).

Holoparasitic plants depend on their hosts for water, minerals, and carbon source. Recent lines of evidence suggest that the parasite's growth depends heavily on compounds that come from the host (Abbes et al., 2009; Basheer et al., 2024; Flores-Sánchez and Garza-Ortiz, 2019; Kumar and Amir, 2021; Kumar et al., 2022; Li et al., 2021). The parasites of Orobanchaceae belong to

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

phloem-feeding holoparasites that have direct symplastic connections between their cells and the sieve elements of their hosts, absorb a wide range of sugars and nitrogen-derived metabolites directly from the phloem of their hosts (Aly et al., 2009; Irving and Cameron, 2009). Indeed, recent studies determined that the metabolic profiling of *P. aegyptiaca* and field dodder (*Cuscuta campestris* Yunck.; syn. *Cuscuta pentagona* Engelm. var. *pentagona*) that parasitized different hosts showed significant changes in the levels of these metabolites (Kumar and Amir, 2021; Kumar et al., 2022; Basheer et al., 2024). The results strengthen the assumption that parasites depend highly on the host's metabolism. However, variations were found in the metabolites profile of their organs (Hacham et al. 2016; Kumar and Amir, 2021; Nativ et al. 2017). This suggests that the parasitic plants can adjust their metabolic profiles to meet the specific needs of each of their organs. This assumption was supported by genome analyses, which showed that they have genes encoding enzymes that can potentially synthesize most of their metabolites (Westwood et al. 2012). Moreover, the finding that the metabolic profiles of the parasitic plants and their hosts differ suggests that the parasites can modify and synthesize their metabolites according to their own needs (Clermont et al., 2019; Hacham et al., 2016; Nandula et al., 2000; Wakabayashi et al., 2015; Westwood, 2013). Studies that measured metabolic interactions between parasites and their hosts raised the possibility that the parasite changes the host's metabolites and hormones. For example, it is found that the host becomes enriched in amounts of particular nitrogenous compounds required by the parasite (Pageau et al., 2003), suggesting that the parasite can influence the host metabolism in a way that promotes export toward the parasite. Some evidence also raised the possibility that the parasite changes in addition to metabolites and the host hormones (Irving and Cameron, 2009).

The environmental conditions and other ecological parameters were also found to change the biomass and the growth of the holoparasites. These parasitic plants are generally most damaging in areas where soils are poor in nutrients (Rubiales et al., 2009). It is reported that the enrichment of the soil with nitrogen causes reduced development and biomass of the parasites (Cechin and Press, 1993; Igbinnosa, I., Thalouarn, 1996) and affects their metabolic profiles (Westwood, 2013). These findings suggest that environmental factors can significantly impact parasitic plants. Indeed, the metabolic profiling was significantly changed when *P. aegyptiaca* developed on the same host that grew in different habitats (Basheer et al., 2024; Kumar et al., 2022).

Managing broomrape plants remains difficult since only a few herbicides are available for use against them (Fernández-Aparicio et al. 2016; Habimana et al. 2014). Glyphosate is one of the most effective herbicides in controlling *P. aegyptiaca*. This non-selective herbicide can inhibit 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS), the key enzyme in the shikimate pathway and the aromatic amino acids biosynthesis pathway enzyme (Dor et al. 2017; Shilo et al. 2017). In addition, imidazolinones that inhibit acetohydroxamic synthase, the key enzyme in the biosynthesis

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

of branched-chain amino acids in plants, can control several *Orobanchaceae* species (Dor et al. 2017). Indeed, enzymatic activities in the biosynthesis of branched-chain and aromatic amino acids were detected in *P. aegyptiaca* (Dor et al., 2017; Shilo et al., 2017). Since these herbicides control enzymes in the biosynthesis pathways of amino acids, they showed that it was necessary to determine the metabolic profiling of the parasites and understand their metabolism. This understanding could contribute to defining additional targets for herbicides capable of controlling these parasites. Therefore, one of the first stages is to determine their primary metabolic profiling. Such knowledge could also contribute to accumulating data on the biology and function of holoparasitic plants (Fernández-Aparicio et al. 2016).

This study aimed to make a comparison between primary metabolic profiles of four different broomrape types: two members of the *Phelipanche* genus (*P. aegyptiaca* and *P. ramosa*) and two members of the *Orobanche* genus (*O. cernua* and *O. cumana*). While the primary metabolic profiling of *P. aegyptiaca* has been previously studied (Clermont et al. 2019; Hacham et al. 2016; Kumar et al. 2022; Nativ et al. 2017), that of the other three holoparasites is yet mostly unknown. That analysis can gain more knowledge about variations of the metabolites in these four parasites. To compare the metabolic profiles of the four parasites, and since the host significantly affects the metabolic profile (Basheer et al., 2024; Kumar and Amir, 2021; Kumar et al., 2022), we tested the metabolic profiling of different *Orobanche* and *Phelipanche* species that parasitize the same host plant, tomato. The results showed that the metabolisms of two species of *Orobanche* have relatively similar profiles that differ from the species belonging to *Phelipanche*. The variations among the four species suggest that each parasite utilizes the metabolites derived from the host differently for their growth and development. In addition to the basic knowledge of the primary metabolic profiles of these parasites, the results could be implemented in the future to search pathways and enzymes that can used as targets for herbicides against all four parasites.

Materials and Methods

Plant material

Tomato cultivar M82 seeds were obtained from Tarsis Agricultural Chemicals Ltd., Israel. Broomrape seeds were collected from *P. aegyptiaca*, *P. ramosa*, *O. cernua* and *O. cumana* inflorescences that parasitized tomato grown in Kibbutz Bet Ha'shita, potato grown in the Golan Heights, tomato grown in Bet Dagan and sunflower grown in the Jezreel Valley, respectively. The seeds were stored and held in the dark at 4°C until use.

Experimental design

M82 tomato seedlings were planted in two-liter pots (Tefen Nachsholim, Israel) using medium-heavy clay-loam soil containing a dry weight basis of 55% clay, 23% silt, 20% sand, 2% organic matter, pH 7.1 (one plant per pot), and slow-release fertilizer at a concentration of 0.6% (w/v)

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

(Osmocote, Scotts Miracle-Gro, Marysville, OH, USA). Each batch of seeds of *P. aegyptiaca*, *P. ramosa* and *O. cernua* was mixed with growth soil at a concentration of 15 ppm. The inoculated soils were transferred to pots, and tomato seedlings were planted. The exception was *O. cumana,* which does not have tomato as its host under field conditions (Sisou et al. 2021). The seeds of *O. cumana* were surface sterilized in 70% ethanol for 1.5 min and then in 1% sodium hypochlorite containing 0.02% (v/v) Tween 20 for 12 min. After thoroughly rinsing the seeds with sterile distilled water, they were distributed evenly on GF/A paper (8x8 cm, ca. 70 seeds/cm^2) in large Petri dishes. The Petri dishes were kept at 25°C for one week, and then 250 µl (5 ppm) of the synthetic stimulant GR24 was added. The paper carrying the seeds was covered with an identical GF/A paper, and the two pieces were stapled together tightly along the edges. The bags described above were inserted into the pots with a tomato plant adjoining the roots. Two bags were placed in each pot.

Control pots did not contain broomrape seeds. The pots were placed in a net house and drip-irrigated as needed. Ten weeks after planting, when the parasites emerged from the soil and the tomato plants had flowers, the broomrapes were harvested from nine tomato plants grown in broomrape-infested soil for each line of the parasite. To harvest all the developmental stages of the parasite, the host's roots were washed gently with tap water to remove the soil and dried on a paper towel. To measure the numbers and biomasses of the parasites, broomrapes from all developmental stages were collected from each pot (Fig. 1).

For the primary metabolic profiling, the parasites were collected from each pot at the same developmental stage, when the plants emerged from the soil and had floral bunds. The sample included all parts of the plant (adventitious roots, lower and upper stems, and floral buds). Therefore, the comparison between these species was based on all of the plant's sections (as previously reported for *P. aegyptiaca*; Nativ et al. 2017). The samples were frozen in liquid nitrogen and lyophilized. The lyophilized tissues were ground to a fine powder by mortar and pestle and kept at -80°C until analysis.

Extraction of primary metabolites using GC-MS

The broomrape samples were homogenized using a Restch MM 301 homogenizer. Twenty mg of the dry weight (DW) fine powder was mixed in 1000 µL of methanol/chloroform/double-distilled water (DDW) (2.5:1:1) with norleucine (4.6 µL of 2 mg per mL) as an internal standard. To determine retention time indices, 7 µL of n-alkane mixture (C12, C15, C19, C22, C28, C32 and C36, 2 µL mL, 1 µL of each in pyridine) was added. After a short vortexing and centrifugation cycle (10 min, 20,000 g at 4°C), 1000 µL of the supernatant was collected in a new tube (Kumar et al. 2022). Three hundred µl of chloroform and 300 µl of double-distilled water were added to the supernatant. Following centrifugation (10 min, 20,000 g at 4°C), 300 µl was taken from the upper

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

polar phase and dried in the speed-vac. Forty µl methoxyaminhydrochlorid (20 mg/ml in Pyridin) was added, and samples were incubated at 37°C with constant agitation. After 2 h, acidic protons were trimethylsilylated by adding 110 µL N-methyl-N-(trimethylsilyl) triflouroacetamide and incubated with constant agitation for 30 min at 37°C. After a short centrifugation cycle, the solution was transferred to GC-MS running vials (Kumar et al. 2022). The single-ion mass method was used for soluble amino acid determination with the BP5MS column (SGE, Trajan: 30 m, 0.25 mm i.d., 0.25 µm thicknesses), while the total-ion-count method was used for the metabolic profiling and separation using the VF-5ms capillary column (Agilent; 30 m + 10 m guard, 0.25-mm i.d., 0.25 mm thicknesses). All analyses were carried out on a GC-MS system (Agilent 7890A) coupled with a mass selective detector (Agilent 5975c) and a Gerstel multipurpose sampler MPS2 (Kumar et al. 2022). Peak finding, peak integration, and retention time correction were measured with the Agilent GC/MSD Productivity ChemStation package (www.agilent.com). Peak areas were normalized to the integral standard (norleucine) signal. Identification of the metabolites (amino acids, sugars, and TCA cycle) was verified using standards.

Total soluble protein determination

For total soluble protein determination, 100 mg fresh weight of broomrape was ground in 400 µl buffer phosphate pH=7.8 with a protease inhibitor cocktail (Sigma). After two centrifugation cycles (20,800 g 14,000 rpm for 20 min), total protein was determined using a Bradford reagent (Bio-Rad Hercules, California, USA) in three concentrations. Bovine serum albumin was used as standard.

Statistical analyses

Four biological replicates of each broomrape type were taken from the different pots for the metabolites study. Statistical significance was evaluated using JMP software version 8.0 (SAS Institute Inc., Cary, NC, USA). Significant differences between broomrape types were calculated according to the Tukey-Kramer HSD test ($p<0.05$) with the one-way ANOVA test. Principal component analysis (PCA) and a heat-map of GC-MS data were plotted using the MetaboAnalyst 5.0 comprehensive tool (http://metaboanalyst.ca/; (Xia et al. 2015) with auto-scaling manipulations. Graphs were compiled using GraphPad Prism 9 scientific software (http://www.graphpad.com).

Results and Discussion

Sample collections and physiology measurements

To gain more knowledge about the variations in primary metabolites of the four *Orobanchaceae* species, two members of the *Phelipanche* genus (*P. aegyptiaca* and *P. ramosa*) and two members of the *Orobanche* genus (*O. cernua* and *O. cumana*) were infected the same host, tomato plants. The host plants were grown in pots containing the same amount of seeds from each parasite. To eliminate the effect of the environment on the metabolic profiling, all the pots were grown under the same conditions, and the parasitic plants were harvested on the same day. The representative photos

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

of the developmental stages of the parasites clearly show that the two species belonging to the *Phelipanche* genus contain secondary branches that branch off from the central stem, as well as long adventitious roots in comparison to the *Orobanche* species (Fig. 1A). The largest number and weight of parasitic plants were detected in *P. aegyptiaca* and *O. cernua*, while *O. cumana* had the lowest values (Fig. 1B). The data are consistent with the knowledge that tomato is not a natural host for *O. cumana* (Sisou et al. 2021), but is one of the favorite hosts for *P. aegyptiaca* (Hershenhorn et al. 2009).

Overview of the primary metabolic profiles analysis of the four parasites

Global primary metabolic profile analyses were performed using Gas Chromatography-Mass Spectrometry (GC-MS) to gain more knowledge about the metabolic differences between the different broomrape types. These analyses enabled us to detect 65 metabolites belonging to eight distinct groups: amino acids (19), sugars (11), sugar acids (6), polyols (5), tricarboxylic acid cycle (TCA) intermediates (4), organic acids (7), fatty acids (10) and others (3) (Supplemental Table S1). Similar results were also reported for *P. aegyptiaca* (Hacham et al., 2016; Kumar et al., 2022; Nativ et al., 2017) and *Cuscuta campestris* (Kumar and Amir, 2021). A principal component analysis (PCA) that is normally used to discriminate between differences in samples composed of multivariable datasets via variance maximization and dimension reduction (Xia et al. 2015) was performed. PC1 (29.2%) and PC2 (24.4%) accounted for 53.6% of the total variance in broomrape types (Fig. 2A). The analysis showed clear separations between the *Phelipanche* and *Orobanche* genera on PC2 (Fig. 2A). This indicates that in addition to morphological characteristics and phylogenetic data (Joel 2009; Delavault 2015), these two genera are also separated based on their metabolic profiling. A smaller separation was also seen between the two species of *Phelipanche*, *P. aegyptiaca* and *P. ramosa*, or between those belonging to *Orobanche*, *O. cernua*s, and *O. cumana*, as they were clearly separated by PC1 (Fig. 2A). This suggests that each species has its own metabolic profile.

The PCA analysis suggests that the primary metabolic profiling of the parasite species differs. A PCA biplot was generated to determine which of the detected metabolites contributed to the variance between the four broomrape species grown on tomato. Variables of metabolites such as gluconic acid, succinic acid, malic acid, nicotinic acid, erythronic acid, phosphoric acid, and glycine were highly associated with PC1. Therefore, they mostly contributed to the variance between *P. ramosa* and *P. aegyptiaca*, as well as between *O. cernua* and *O. cumana* (Fig. 2B). PC2 differentiated between several organic acids such as shikimate, quinic acid, citric acid and fumaric acid, as well as sugars (cellobiose, fucose), amino acids (valine, cysteine, leucine, tryptophan) and fatty acid (C18:1, C18:2 C18:3 and C20:1) (Fig. 2B). PC2 mainly separated between the two species of *Phelipanche* and of *Orobanche* (Fig. 2B; Supplemental Fig. S1).

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

According to the biplot analysis, certain metabolites that appeared as the main factors contributing to the variation between the four parasitic plants differed from those contributing to the difference between *P. aegyptiaca* developed on ten different hosts (Kumar and Amir, 2022). In the latter case, sugars, phosphoric acid, shikimate, and polyols are the main factors influencing the variance in the parasite's metabolites (Kumar and Amir, 2022). This is also similar to the results of biplot analyses made from the *C. campestris* that developed on three different hosts (Kumar and Amir, 2021).

Detailed metabolic data of the four parasites

A comparison between the four species using a heat-map analysis shows that *P. aegyptiaca* and *O. cumana* had relatively higher levels of most of the metabolites. In contrast, only a few metabolites were relatively high in *O. cernua* (glycine, lysine, asparagine, C18:2, C16:0, quinic acid and putrescine) (Fig. 3). Compared to the other parasites, *P. ramosa* had the lowest contents of most of the metabolites detected (Fig. 3).

To obtain more information about the differences, each metabolite was analyzed separately. Among the 19 free amino acids that were detected, 12 did not differ significantly between the broomrape types (Fig. 4). The levels of five amino acids were significantly higher in *P. aegyptiaca* compared to *O. cumana*, including the branched amino acids (valine, leucine, and isoleucine), tryptophan and cysteine. Of these amino acids, only leucine, tryptophan, and cysteine levels were higher in *P. aegyptiaca* compared to *O. cernua*. The levels of the other two amino acids, proline and ornithine, were high in *O. cumana* compared to *P. aegyptiaca*, *P. ramosa* and *O. cernua* (Fig. 4A). Significant changes in the levels of amino acids were also detected in Japanese dodder (*Cuscuta japonica* Choisy) infected host and non-host plants (Guo et al., 2022). Differing levels of amino acids can affect protein content. It was previously shown that even a change in the level of one soluble amino acid can affect the total protein content. Examples are higher soluble levels of each of these soluble amino acids, methionine, valine, isoleucine or cysteine, which led to elevated total protein content in different plants (Hacham et al. 2017; Nguyen et al. 2012; Sim et al. 2021; Sun et al. 2023). A Bradford analysis was performed on the soluble protein fraction extracted from the parasites to examine this assumption. This analysis revealed that protein content was significantly higher in *P. aegyptiaca*, followed by *P. ramosa*, whereas *O. cumana* and *O. cernua* showed a significantly lower content (Fig. 4B). The results are in accordance with the finding that *P. aegyptiaca* has higher levels of five soluble amino acids compared to *O. cumana* (Fig. 4A). A previous study revealed that *P. aegyptiaca* had a 9.8-fold higher protein content compared to roots of the tomato host (Hacham et al. 2016). Overall, the finding shows that two *Phelipanche* species (*P. aegyptiaca* and *P. ramosa*) had higher protein contents of about 3- to 5-fold compared to the

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

Orobanche species (*O. cumana* and *O. cernua*), which corresponds to the levels of at least several amino acids.

Of the 11 sugars detected, the levels of seven changed significantly between the broomrape types. *O. cumana* had high levels of four sugars – mannose, trehalose, xylose, and cellobiose – compared to the other three broomrapes (Fig. 5). *P. aegyptiaca* had a higher level of fucose than the other three broomrapes, while the levels of fructose, mannose, and ribose were higher than *O. cernua*. *P. ramosa* had higher levels of mannose compared to *O. crenua*. The levels of sugar acids and polyols that varied among the four broomrape types (erythronic acid, gluconic acid, lactic acid, threonic acid, glycerol, myo-Inositol, sorbitol, and erythritol) were higher for *O. cumana* compared to *O. crenata*. *P. aegyptiaca* had lower levels of threonic acid and sorbitol compared to *O. cumana* and similar levels to *O. cumana* of the rest. *P. ramosa* had low levels of gluconic acid, threonic acid, myo-Inositol, sorbitol and erythritol compared to *O. cumana* (Fig. 5). The four TCA metabolites that were detected by GC-MS showed significant changes between the broomrape types. *O. cumana* and *P. aegyptiaca* had higher levels of malate and succinate than the other two broomrapes; in addition, the level of citrate was higher in *O. cumana*, and the level of fumarate was higher in *P. aegyptiaca* (Fig. 5). *P. ramosa* had lower levels of all four TCA metabolites. In comparison, *O. cernua* had lower levels of fumarate, malate, and succinate (Fig. 5). Ten fatty acids were detected, seven of which showed significant changes between the four parasites (Fig. 6). The rest of the metabolites exhibited a comparatively large variety between the four broomrape types. Shikimate and quinic acid were higher in *O. cumana* and *O. cernua* compared to *P. aegyptiaca* and *P. ramosa*. Butanedioic acid was higher in *O. cumana* (Fig. 6).

Of the 65 metabolites identified, 26 did not change significantly between the four broomrapes species. They included 12 amino acids, four sugars, two sugar acids, one polyol, two organic acids, three fatty acids, and two others (Figs. 4-6; Supplemental Table S1). The results show that despite several similarities, most metabolites changed in each of the examined parasites, and each had its own metabolic characterization. This suggests they have varying capacities for producing or accumulating metabolites according to their needs. However, the PCA and biplot data suggest that the metabolism of the parasitic plants is also related to the genus and less to the host.

Final remarks

The main objective of the current study was to determine the primary metabolic profiling of four parasitic plants belonging to two different genera of the *Orobanchaceae* family. Previous studies showed that the host has a significant impact on the primary metabolites of the parasitic plants (Basheer et al., 2024; Kumar and Amir, 2021; Kumar et al., 2022). Therefore, to enable comparison in the current study, the parasite plants analyzed were grown on the same host.

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

The results show significant metabolic differences between two species of two genera of *Orobanche* and *Phelipanche* (Figs. 2-6), implying that in addition to morphological and other genetic markers, these two genera can also be distinguished by their metabolic profiles. However, this assumption requires further study to reveal the metabolic profiles of other species belonging to these genera. The results also show that the two species in each genus had different metabolic profiles. For example, *O. cumana* had significantly higher levels of sugars, sugar acid, polyols, TCA-organic acid, and several fatty acids than *O. cernua* (Figs. 5-6). These two species were considered closely related; however, molecular techniques clearly distinguished them, separating them into different species (Delavault, 2015; Delavault and Thalouarn, 2002; Piwowarczyk et al. 2018).

The results of this study show that the metabolic profiles of the four parasitic plants tested differed significantly. Based on the results obtained in the current study and those of our previous studies (Hacham et al. 2016; Kumar and Amir, 2021; Kumar et al. 2022; Nativ et al. 2017), we can strongly suggest that each of the parasites absorbs the host's metabolites but also can self-regulate their metabolites, some of which were accumulated and others were catabolized to synthesize other metabolites. The ability of the parasite to form most metabolites was suggested by the finding that they have genes for enzymes that could theoretically lead to the synthesis of most of their metabolites (Westwood et al. 2012). Other studies showed that some of their enzymes are active, such as those related to sugar, amino acids, and carotenoids syntheses (e.g., Abbes et al. 2009; Dor et al. 2017; Emran et al. 2020; Farrokhi et al. 2019, 2021; Shilo et al. 2017; Wakabayashi et al. 2015).

This study provides more data about the primary metabolic profiles of these four parasites. Previously, other studies reported the content of several metabolites, such as some fatty acids, alkenes, ketones, fatty alcohols, and polyols, which were described when they were associated with secondary metabolites (reviewed by Scharenberg and Zidorn, 2018). Several other studies were also performed on the host's metabolites after infection by the parasite (e.g., En-nahli et al. 2021; Nandula et al. 2000; Rispail et al. 2007; Wakabayashi et al. 2015). Yet, knowledge about the diversity of the parasitic plant's primary metabolism remains poorly understood, and the current study enhances our knowledge about this metabolism. Such knowledge could potentially support additional physiological and molecular-genetic studies and define more specific targets for herbicides that control these parasites (Fernández-Aparicio et al., 2016). These targets could be enzymes in the metabolism that exist extensively in the parasitic plants but less in the hosts, for example, the enzymes involved in mannitol synthesis, whose level is significantly higher in *P. aegyptiaca* than in its host (Hacham et al. 2016) and had no significant changes in the other three species (Fig. 5). Indeed, one of these enzymes was previously suggested to be a target to control *P.*

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

aegyptiaca (Aly et al., 2009). Another approach is to identify enzymes within the metabolic pathways of parasitic plants whose gene or protein sequences are distinct from those of their hosts. This could lead to choosing selective parasite herbicides. The enzymes can be selected from pathways where the metabolite levels did not change significantly between the four parasitic plants. Such herbicides can damage all the four parasites. These pathways include those that produced different sugars such as galactose, amino acids such as serine, glutamate, threonine, and methionine, and fatty acids such as palmitate and docosanoate. In the future, their genes and enzymes could be tested to determine if they are targets for such selective herbicides. In addition to herbicides, it would be worth checking the application of other biotechnological strategies using the CRISPR/Cas9 system and T-DNA insertions for engineering resistance against parasitic plants (Aly et al., 2021).

Acknowledgments

We would like to thank Janet Covaliu for the English editing of this manuscript.

Funding

This work was supported by a grant from the Ministry of Agriculture & Rural Development (Grant no. 21-01-0036).

Competing Interests

The authors have no competing interests to declare that are relevant to the content of this article. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

References

Abbes Z, Kharrat M, Delavault P, Chaïbi W, Simier P (2009) Osmoregulation and nutritional relationships between Orobanche foetida and faba bean. Plant Signal Behav 4: 336–338

Aly R, Cholakh H, Joel DM, Leibman D, Steinitz B, Zelcer A, Naglis A, Yarden O, Gal-On A (2009) Gene silencing of mannose 6-phosphate reductase in the parasitic weed Orobanche aegyptiaca through the production of homologous dsRNA sequences in the host plant. Plant Biotechnol J 7:487–498

Aly R, Matzrafi M, Bari VK (2021) Using biotechnological approaches to develop crop resistance to root parasitic weeds. Planta 253: 1–11

Basheer L, Niv D, Cohen A, Gutman R, Hacham Y, Amir R (2024) Egyptian broomrape (Phelipanche aegyptiaca): from foe to friend? Evidence of high nutritional value and potential suitability for food use. Future Food 10: 100413

Casadesús A, Munné-Bosch S (2021) Holoparasitic plant-host interactions and their impact on Mediterranean ecosystems. Plant Physiolo185: 1325–1338

Cechin I, Press MC (1993) Nitrogen relations of the sorghum-Sfriga hermonthica hostparasite association: growth and photosynthesis. Plant, Cell Environ 16: 237–247

Clermont K, Wang Y, Liu S, Yang Z, dePamphilis CW, Yoder JI (2019) Comparative metabolomics of early development of the parasitic plants Phelipanche aegyptiaca and Triphysaria versicolor. Metabolites 9: 114-133

Cochavi A, Rapaport T, Gendler T, Karnieli A, Eizenberg H, Rachmilevitch S, Ephrath EJ (2017) Recognition of Orobanche cumana below-ground parasitism through physiological and hyper spectral measurements in sunflower (Helianthus annuus L.). Front Plant Sci 8: 1–12

Delavault P (2015) Knowing the parasite: Biology and genetics of Orobanche. Helia 38: 15–29

Delavault P, Thalouarn P (2002) The obligate root parasite Orobanche cumana exhibits several rbcL sequences. Gene 297: 85–92

Dor E, Galili S, Smirnov E, Hacham Y, Amir R, Hershenhorn J (2017) The effects of herbicides targeting aromatic and branched chain amino acid biosynthesis support the presence of functional pathways in broomrape. Front Plant Sci 8: 1–15

Emran S, Nawade B, Yahyaa M, Abu Nassar J, Tholl D, Eizenberg H, Ibdah M (2020) Broomrape infestation in carrot (Daucus carota): Changes in carotenoid gene expression and carotenoid accumulation in the parasitic weed Phelipanche aegyptiaca and its host. Sci Rep 10: 2–11

En-nahli Y, El Arroussi H, Kumar S, Bouhlal O, Mentag R, Hejjaoui K, Douaik A, Abbes, I, Eddine N, Es-Safi I, Amri M (2021) Resistance to Orobanche crenata Forsk. in lentil (Lens

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

culinaris Medik.): exploring some potential altered physiological and biochemical defense mechanisms. J Plant Interact 16: 321–331

Farrokhi Z, Alizadeh H, (2019) Developmental patterns of enzyme activity, gene expression, and sugar content in sucrose metabolism of two broomrape species. Plant Physiol Biochem 142: 8–14

Farrokhi Z, Alizadeh H (2021) Egyptian broomrape sucrose metabolism in response to different host plants. Weed Res 61:137–145

Fernández-Aparicio, M, Reboud X, Gibot-Leclerc S (2016) Broomrape weeds. Underground mechanisms of parasitism and associated strategies for their control: A review. Front Plant Sci 7: 21-25

Flores-Sánchez IJ, Garza-Ortiz A (2019) Is there a secondary/specialized metabolism in the genus Cuscuta and which is the role of the host plant? Phytochem Rev 18: 5-9

Guo C, Qin L, Ma Y, Qin J (2022) Integrated metabolomic and transcriptomic analyses of the parasitic plant Cuscuta japonica Choisy on host and non-host plants. BMC Plant Biol 22: 1–16

Habimana S, Nduwumuremyi A, Chinama RJD (2014) Management of orobanche in field crops-a review. J Soil Sci and Plant Nut 14: 43–62

Hacham Y, Hershenhorn J, Dor E, Amir R (2016) Primary metabolic profiling of Egyptian broomrape (Phelipanche aegyptiaca) compared to its host tomato roots. J Plant Physiol 205: 11–19

Hacham Y, Matityahu I, Amir R (2017) Transgenic tobacco plants having a higher level of methionine are more sensitive to oxidative stress. Physiol Plant 160: 242–252

Hershenhorn J, Eizenberg H, Dor E, Kapulnik Y, Goldwasser Y (2009) *Phelipanche aegyptiaca* management in tomato. Weed Res 49: 34–47

Igbinnosa I, Thalouarn P (1996) Nitrogen assimilation enzyme activities in witchweed (Striga) in hosts presence and absence. Weed Sci 44: 224–232

Irving LJ, Cameron DD (2009) Chapter 3. You are What You Eat. Interactions between root parasitic plants and their hosts. Adv Bot Res 50: 87-138

Joel DM (2007) Direct infection of potato tubers by the root parasite *Orobanche aegyptiaca*. Weed Res 47: 276–279

Joel DM (2009) The new nomenclature of Orobanche and Phelipanche. Weed Res. 49: 6–7

Kumar K, Amir R (2021) The effect of a host on the primary metabolic profiling of *Cuscuta campestris*' main organs, haustoria, stem and flower. Plants 10: 1–13

Kumar K, Hacham Y, Amir R (2022) The Effect of 10 crop plants that served as hosts on the primary metabolic profile of the parasitic plant *Phelipanche aegyptiaca*. Metabolites 12:

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

1195- 1210

Li L, Lietz G, Seal CJ (2021) Phenolic, apparent antioxidant and nutritional composition of quinoa (*Chenopodium quinoa* Willd.) seeds. Internat J Food Sci Technol 56: 3245–3254

Mutuku JM, Cui S, Yoshida S, Shirasu K (2021). Orobanchaceae parasite–host interactions. New Phytol 230: 46–59

Nandula, V. K., Foster, J. G., and Foy, C. L. (2000). Impact of Egyptian broomrape (*Orobanche aegyptiaca* (Pers.) parasitism on amino acid composition of carrot (*Daucus carota* L.). J Agri Food Chem 48: 3930–3934

Nativ N, Hacham Y, Hershenhorn J, Dor E, Amir R (2017) Metabolic investigation of *Phelipanche aegyptiaca* reveals significant changes during developmental stages and in its different organs. Front Plant Sci 8: 491-504

Cuong NH, Hoefgen R, Hesse H (2012) Improving the nutritive value of rice seeds: elevation of cysteine and methionine contents in rice plants by ectopic expression of a bacterial serine acetyltransferase. J Exp Bot 63: 5991–6001

Pageau K, Simier P, Le Bizec B, Robins RJ, Fer A (2003). Characterization of nitrogen relationships between Sorghum bicolor and the root-hemiparasitic angiosperm *Striga hermonthica* (Del.) Benth. using K15NO3 as isotopic tracer. J Exp Bot 54: 789–799

Piwowarczyk R, Denysenko-Bennett M, Góralski G, Kwolek D, Pedraja ÓS, Mizia P, Pedraja SO, Mizia P, Cygan M, Joachimiak A (2018) Phylogenetic relationships within orobanche and phelipanche (orobanchaceae) from central Europe, focused on problematic aggregates, taxonomy, and host ranges. Acta Biol Crac Ser Bot 60: 45–64

Rispail N, Dita MA, González-Verdejo C, Pérez-De-Luque A, Castillejo MA, Prats E, Román B, Jorrín J, Rubiales D (2007) Plant resistance to parasitic plants: Molecular approaches to an old foe: Research review. New Phytol 173: 703–712

Rubiales D, Verkleij J, Vurro M, Murdoch AJ, Joel DM (2009). Parasitic plant management in sustainable agriculture. Weed Res 49: 1–5

Scharenberg F, Zidorn C (2018) Genuine and sequestered natural products from the genus Orobanche (Orobanchaceae, Lamiales). Molecules 23: 2821- 2851

Shilo T, Rubin B, Plakhine D, Gal S, Amir R, Hacham Y, Wolf S, Eizenberg H (2017) Secondary effects of glyphosate action in *Phelipanche aegyptiaca*: Inhibition of solute transport from the host plant to the parasite. Front Plant Sci 8: 1–16

Sim SYJ, Srv A, Chiang JH, Henry CJ (2021) Plant proteins for future foods: A roadmap. Foods 10: 1–31

Sisou D, Tadmor Y, Plakhine D, Ziadna H, Hübner S, Eizenberg H (2021) Biological and transcriptomic characterization of pre-haustorial resistance to sunflower broomrape

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

(*Orobanche cumana* w.) in sunflowers (*Helianthus annuus*). Plants 10: 1810-1824

Sun M, Li S, Yu H, Gong Q, Zhang B, Liu G, Xiao Y, Peng F (2023) Effects of valine and urea on carbon and nitrogen accumulation and lignin content in peach trees. Plants 12: 1596-1619

Wakabayashi T, Joseph B, Yasumoto S, Akashi T, Aoki T, Harada K, Muranaka S, Bamba T, Fukusaki E, Takeuchi Y (2015) Planteose as a storage carbohydrate required for early stage of germination of Orobanche minor and its metabolism as a possible target for selective control. J Exp Bot 66: 3085–3097

Westwood, J. H. (2013) The Physiology of the Established Parasite–Host Association. in Parasitic Orobanchaceae, ed. L. (eds) Joel D, Gressel J Musselman (Springer, Berlin, Heidelberg.), 87–114

Westwood JH, dePamphilis CW, Das M, Fernández-Aparicio M, Honaas LA, Timko MP, Wafula EK, Wickett JN,

Yoder IJ (2012) The parasitic plant genome project: New tools for understanding the biology of Orobanche and Striga. Weed Sci 60: 295–306

Xia J, Sinelnikov IV, Han B, Wishart DS (2015) MetaboAnalyst 3.0-making metabolomics more meaningful. Nuc Acid Res 43:W251–W257

Supplementary Information

Supplemental Table S1: Metabolites peak area as measured by GC-MS. Data shown are means ± SD of four replicates for each plant type. Significance was calculated according to the Tukey-Kramer HSD test ($p<0.05$) and is identified by different small letters.

Supplemental Figure S1: Bioplot analysis applied to the set of 65 identified metabolites. The compounds causing the strongest divergence among the samples were shown. Samples individual replicates are displayed as points while metabolites are displayed as arrows.

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

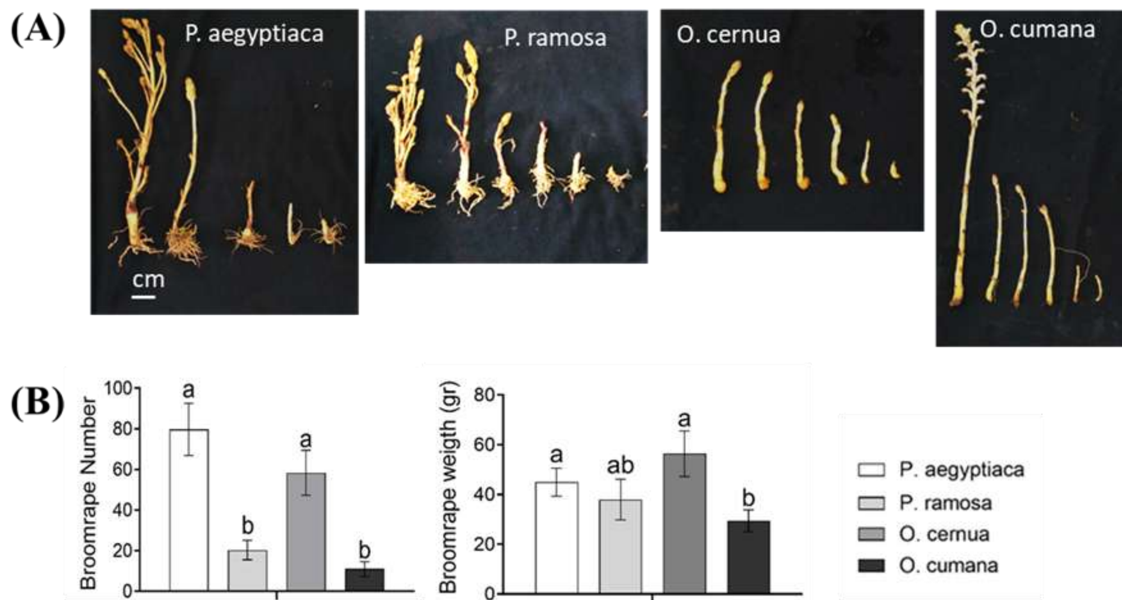

Figure 1. Phenotype of four different broomrape types grown on tomato. (A) The phenotype of the four broomrape species grown on tomatoes. Nine pots were used for each species, and the representative developmental stages are shown for each species. **(B)** Number and weight of different broomrape types per pot. Measurements were taken after the roots of the tomato plants were washed from the soil, and all of the parasitic plants were collected. Data are presented as the mean ± SE of nine different pots. Different letters represent statistical significance ($p \leq 0.05$) using the Tukey-Kramer test.

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

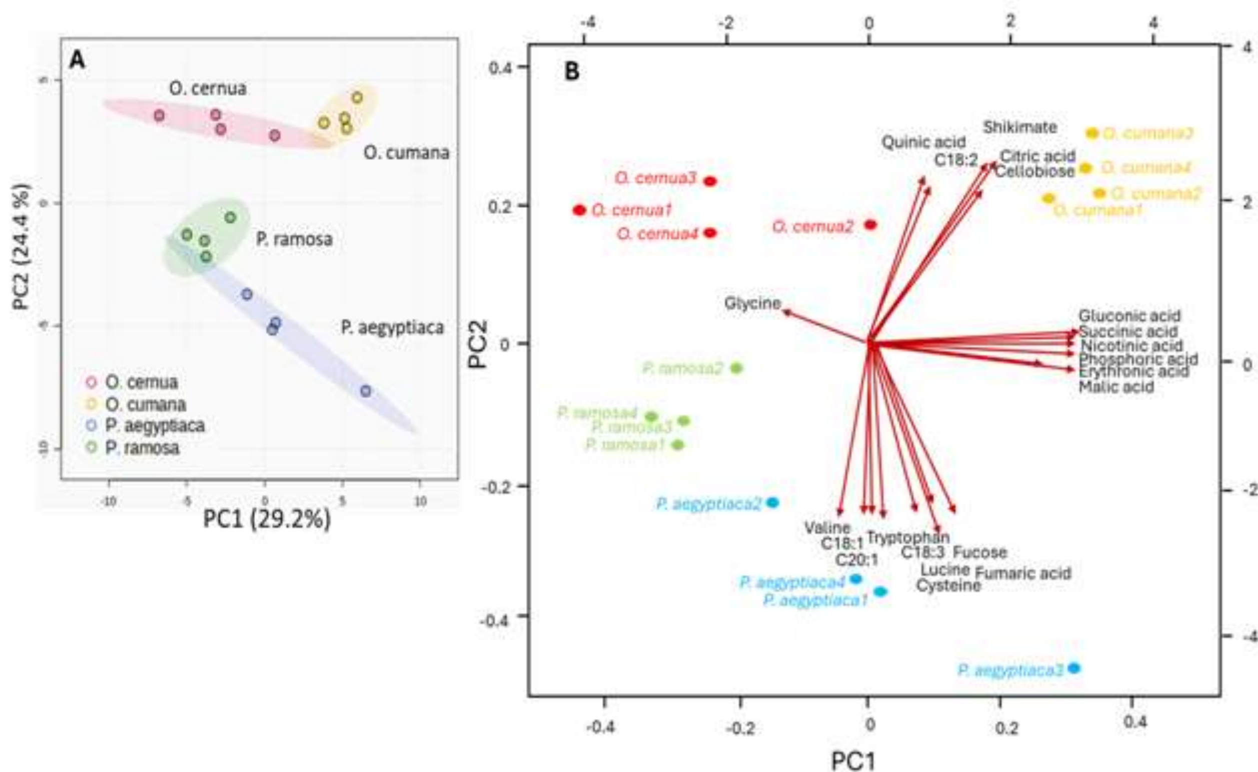

Figure 2. Principal component (PCA) (A) and biplot (B) analyses of data obtained from the GC-MS analysis. The variances of the PCA explained by the first two components (PC1 and PC2) appear in parentheses. The biplot analysis was applied to the set of 65 identified metabolites, showing the compounds causing the strongest divergence among the samples (modified from Supplemental Fig. S1). Samples of individual replicates are displayed as points, while metabolites are displayed as arrows.

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

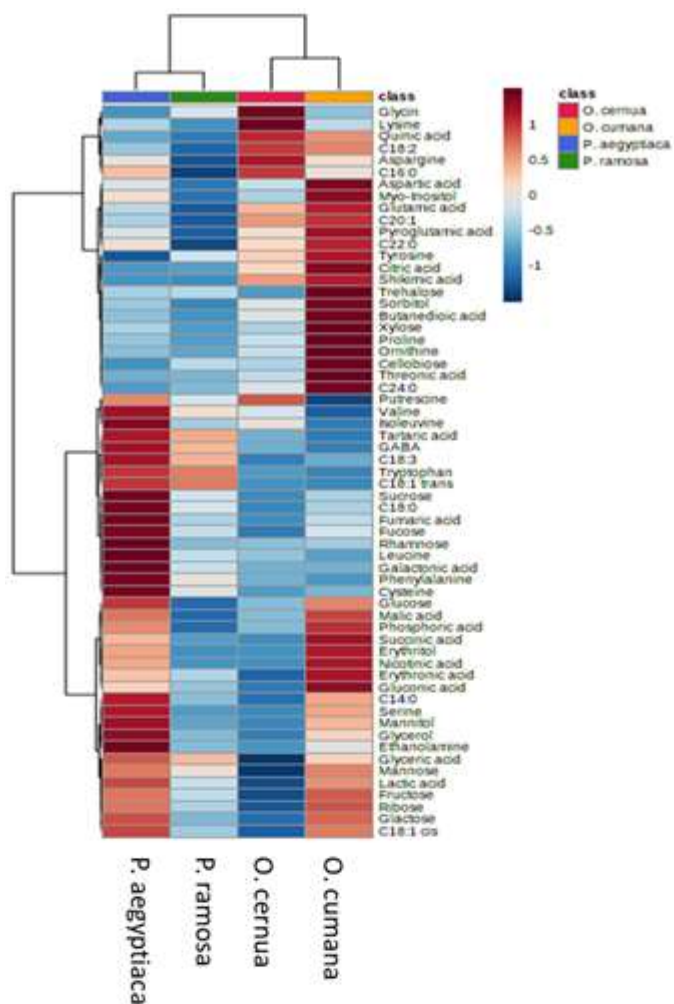

Figure 3. Heat-map of the 65 primary metabolites detected by GC-MS. Distance is measured according to Pearson. The data represent four replicates for each broomrape type.

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

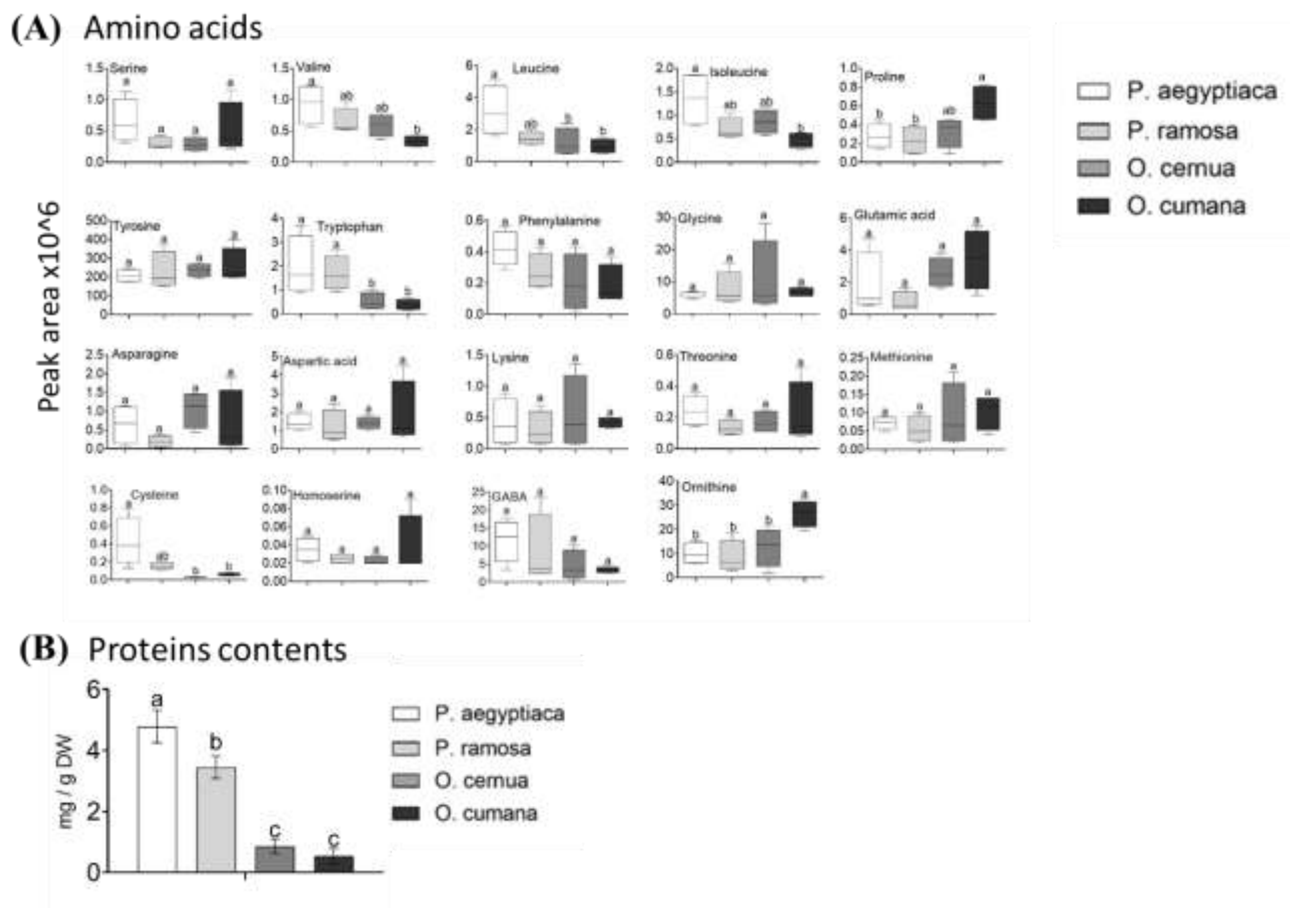

Figure 4. Levels of free amino acids and soluble proteins. (A) Free amino acids as detected by GC-MS. Results are presented on a graph showing a compound level represent the peak area (*10^{6}) normalized to the internal standard (norleucine). **(B)** Total protein contents in the albumin fraction as measured using a Bradford assay. Data shown are means ± SD of four replicates for each type of plant. Significance was calculated according to the Tukey-Kramer HSD test ($p<0.05$) and is identified by different small letters.

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

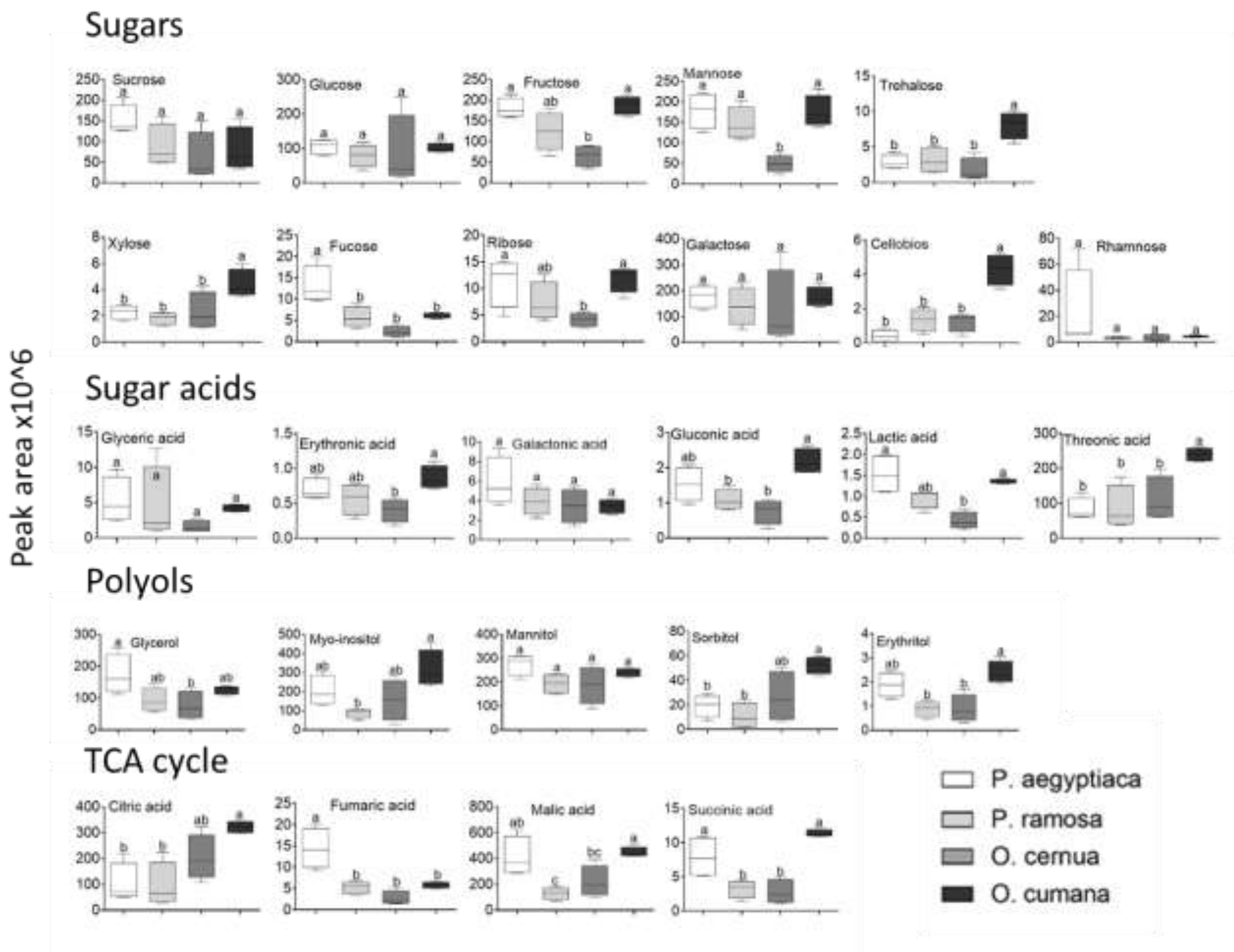

Figure 5. Levels of sugars, sugar acids, polyols, and TCA metabolites as detected by GC-MS. Results are presented representing the levels of these metabolites as the peak area ($*10^{6}$) normalized to the internal standard (norleucine). Data shown are means ± SD of four replicates for each type of plant. Significance was calculated according to the Tukey-Kramer HSD test ($p<0.05$) and is identified by different small letters.

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press

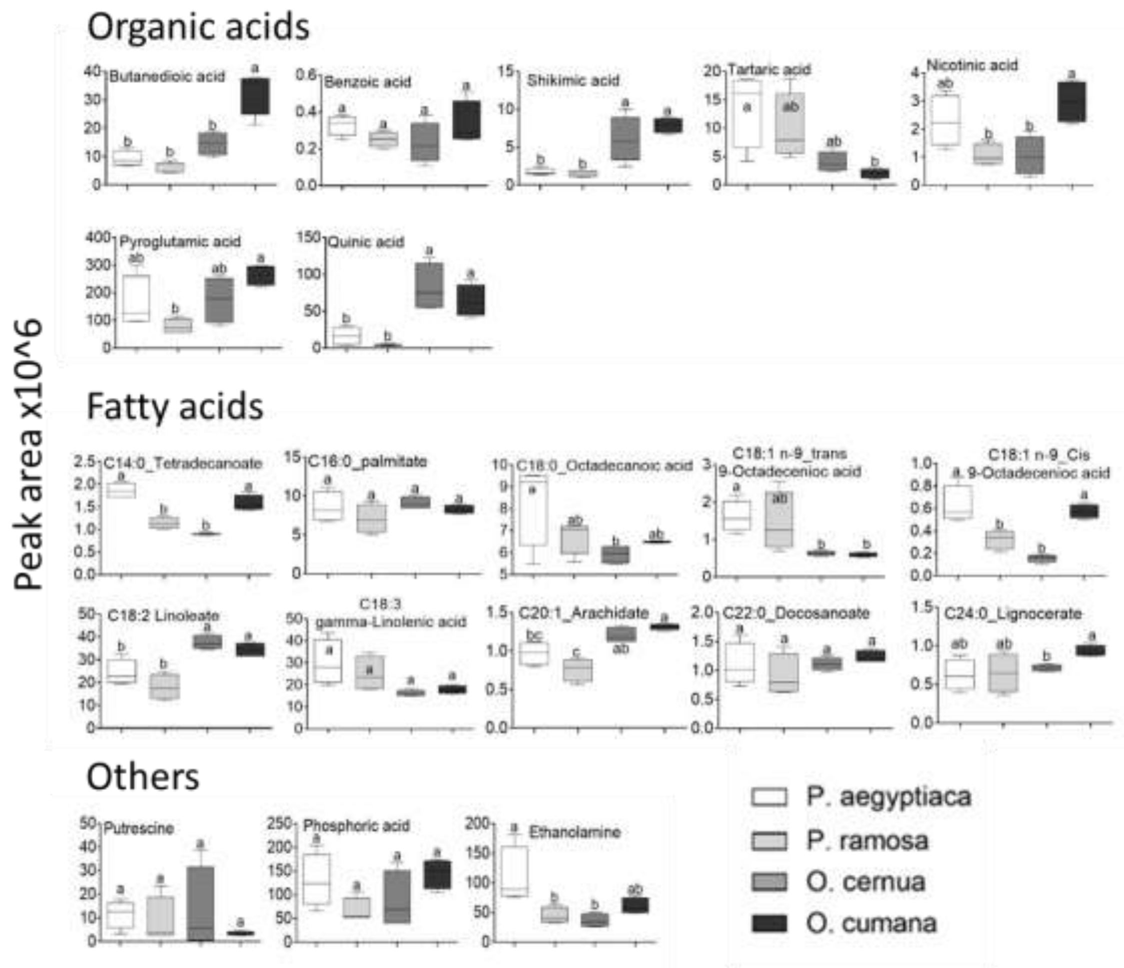

Figure 6. Levels of organic and fatty acids and three other metabolites as detected by GC-MS. Results are presented on a graph representing compound level as the peak area (*10^6) normalized to the internal standard (norleucine). Data shown are means ± SD of four replicates for each type of plant. Significance was calculated according to the Tukey-Kramer HSD test ($p<0.05$) and is identified by different small letters.

https://doi.org/10.1017/wsc.2024.75 Published online by Cambridge University Press