

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

## Research Article

**Cite this article:** Dor E, Guy A, Amir R, Hacham Y (2024) Primary metabolic profiling of four broomrapes belonging to *Orobanche* and *Phelipanche* species. *Weed Sci.* **72**: 714–722. doi: [10.1017/wsc.2024.75](https://doi.org/10.1017/wsc.2024.75)


Received: 6 May 2024  
Revised: 24 July 2024  
Accepted: 8 October 2024  
First published online: 30 October 2024

**Associate Editor:**  
William Vencill, Univ of Georgia-Crop/Soils

**Keywords:**  
GC-MS analysis; holoparasitic plant; primary metabolic profiling

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### 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 Egyptian broomrape [*Orobanche aegyptiaca* Pers.; syn.: *Phelipanche aegyptiaca* (Pers.) Pomel]. This study aimed at obtaining more knowledge about the primary metabolic profiling of four parasite species belonging to the Orobanchaceae family—sunflower broomrape (*Orobanche cumana* Wallr.), *Orobanche cernua*, *P. aegyptiaca*, and branched broomrape [*Orobanche ramosa* L.; syn.: *Phelipanche ramosa* (L.) Pomel.]—that developed on tomato (*Solanum lycopersicum* L.) as a host. Gas chromatography–mass spectrometry analysis demonstrated that significant differences in metabolite content occur between species belonging to *Orobanche* compared with 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.

### 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 broomrapes 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 infested 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.) Pomel]. These parasites parasitize critical crops, thus negatively affecting human nutrition and leading to heavy economic losses (Yoshida et al. 2012). *Phelipanche 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 geographic (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. 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 phloem-feeding holoparasites that have direct symplastic connections between their cells and the sieve elements of their hosts, allowing them to 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 (Basheer et al. 2024; Kumar and Amir 2021; Kumar et al. 2022). The results strengthen the assumption that parasites are highly dependent on their host's metabolism. However, variations were found in the metabolic profiles of their organs (Hacham et al. 2016; Kumar and Amir 2021; Nativ et al. 2017). This suggests that parasitic plants can adjust their metabolic profiles to meet the specific needs of each of their organs. This assumption was supported by genomic analyses, which showed that these plants 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 was 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. Evidence also raised the possibility that the parasite may affect not only the host metabolites but also its 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 and 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, because 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 nonselective herbicide can inhibit 5-enolpyruvyl shikimate-3-phosphate synthase, 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 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). The authors showed that it was necessary to determine the metabolic profiling of the parasites and understand their metabolism, because the herbicides involved control enzymes in the biosynthesis pathways of amino acids. 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), the metabolic profiles of the other three holoparasites are mostly unknown. Such an analysis would provide more knowledge about variations of the metabolites in these four parasites. To compare the metabolic profiles of the four parasites and considering that the host has a significant impact on the parasite's metabolic profile (Basheer et al. 2024; Kumar and Amir 2021; Kumar et al. 2022), we analyzed the metabolic profiles 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 its 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 be used as targets for herbicides against all four parasites.

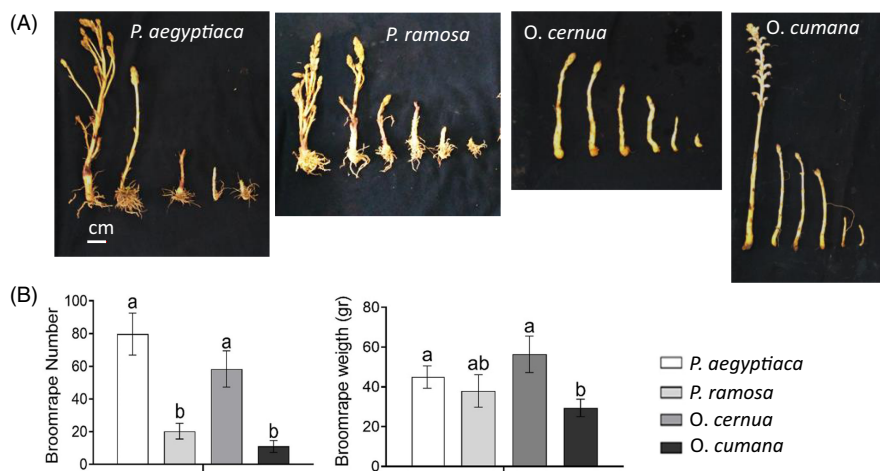
## Materials and Methods

### Plant Material

Tomato 'M82' seeds were obtained from Tarsis (Tarsis Agricultural Chemicals, 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 2-L pots (one plant per pot; Tefen Flow and Dosing Technologies, Kibbutz Nahsholim, 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, and slow-release fertilizer at a concentration of 6 g L<sup>-1</sup> (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



**Figure 1.** Phenotype of four different broomrape types grown on tomato: *Phelipanche aegyptiaca*, *Phelipanche ramosa*, *Orobanche cernua*, and *Orobanche cumana*. (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  $\pm$  SE of nine different pots. Different lowercase letters represent statistical significance ( $P \leq 0.05$ ) using the Tukey-Kramer test.

hypochlorite containing 0.02% (v/v) Tween 20 for 12 min. After a thorough rinsing with sterile distilled water, the seeds were distributed evenly on GF/A paper (8 by 8 cm, ca. 70 seeds  $\text{cm}^{-2}$ ) in large petri dishes. The petri dishes were kept at 25 C for 1 week, and then 250  $\mu\text{l}$  (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. At 10 wk 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 (Figure 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 buds. 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 parts (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 using a mortar and pestle and kept at  $-80$  C until analysis.

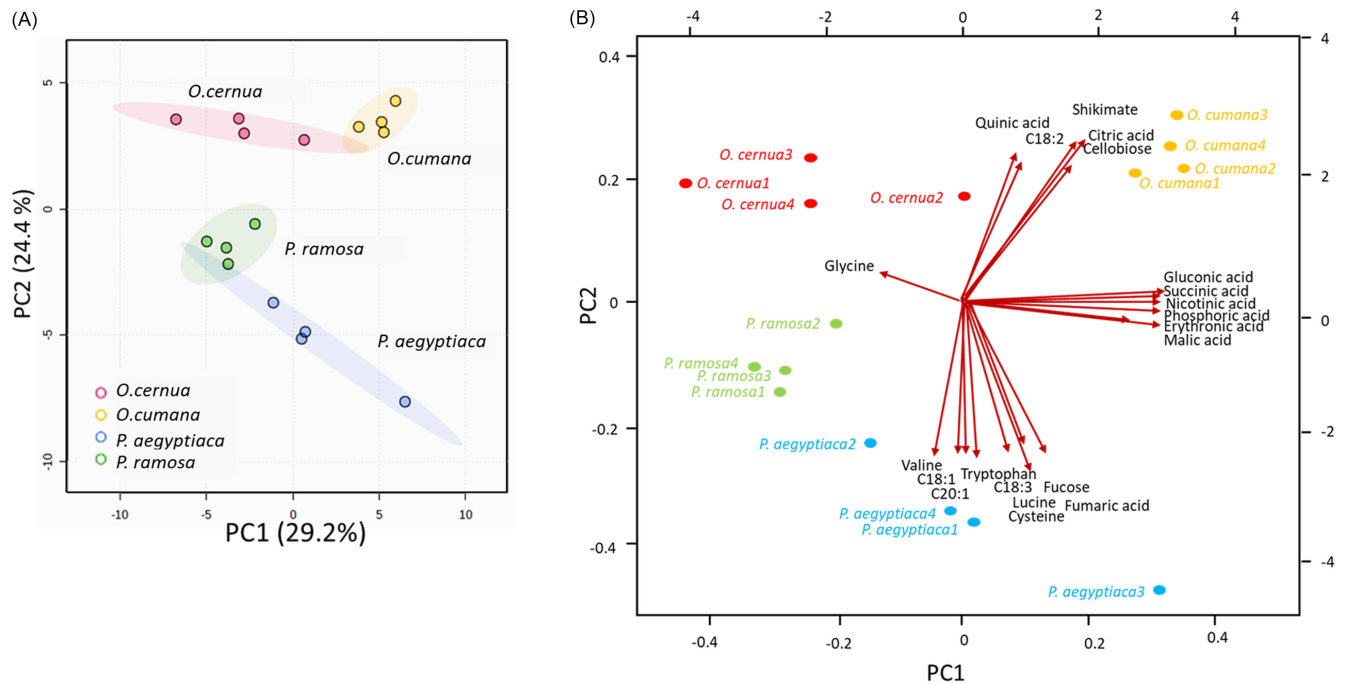
#### Extraction of Primary Metabolites Using Gas Chromatography–Mass Spectrometry (GC-MS)

The broomrape samples were homogenized using a Restch MM 301 homogenizer. Twenty milligrams of the dry weight fine powder was mixed in 1,000  $\mu\text{l}$  of methanol/chloroform/double-distilled water (DDW) (2.5:1:1) with norleucine (4.6  $\mu\text{l}$  of 2 mg  $\text{ml}^{-1}$ ) as an internal standard. To determine retention time indices, 7  $\mu\text{l}$  of *n*-alkane mixture (C12, C15, C19, C22, C28, C32, and C36,

2  $\mu\text{l}$   $\text{ml}^{-1}$  of each in pyridine) was added. After a short vortexing and centrifugation cycle (10 min, 20,000  $\times$  g at 4 C), 1,000  $\mu\text{l}$  of the supernatant was collected in a new tube (Kumar et al. 2022). Three hundred microliters of chloroform and 300  $\mu\text{l}$  of DDW were added to the supernatant. Following centrifugation (10 min, 20,000  $\times$  g at 4 C), 300  $\mu\text{l}$  was taken from the upper polar phase and dried in the speed-vac. Forty microliters of methoxyamine hydrochloride (20 mg  $\text{ml}^{-1}$  in Pyridine) was added, and samples were incubated at 37 C with constant agitation. After 2 h, acidic protons were trimethylsilylated by adding 110  $\mu\text{l}$  *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide 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 a BP5MS column (SGE GC column, Trajan Scientific and Medical, Australia; 30 m, 0.25-mm inner diameter, 0.25- $\mu\text{m}$  thicknesses), while the total ion count method was used for the metabolic profiling and separation using a VF-5ms capillary column (Agilent Technologies, Inc, United States; 30 m + 10-m guard, 0.25-mm inner diameter, 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 (Agilent Technologies) (Kumar et al. 2022). Peak finding, peak integration, and retention time correction were measured with the Agilent GC/MSD Productivity ChemStation package (Agilent). Peak areas were normalized to the integral standard (norleucine) signal. Identification of the metabolites (amino acids, sugars, and tricarboxylic acid cycle [TCA] cycle) was verified using standards.

#### Total Soluble Protein Determination

For total soluble protein determination, 100 mg of fresh weight of broomrape was ground in 400  $\mu\text{l}$  of buffer phosphate (pH 7.8) with a protease inhibitor cocktail (P9599, Sigma Aldrich, an affiliate of Merck KGaA, Darmstadt, Germany). After two centrifugation cycles (20,800  $\times$  g, 14,000 rpm for 20 min), total protein was determined using a Bradford reagent (Bio-Rad, Hercules, CA, USA) in three concentrations. Bovine serum albumin was used as the standard.



**Figure 2.** Principal component (PCA) (A) and biplot (B) analyses of data obtained from the gas chromatography–mass spectrometry (GC-MS) analysis of *Phelipanche aegyptiaca*, *Phelipanche ramosa*, *Orobanche cernua*, and *Orobanche cumana*. 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 Supplementary Figure S1). Samples of individual replicates are displayed as points, while metabolites are displayed as arrows.

### Statistical Analyses

Four biological replicates of each broomrape type were taken from the different pots for the metabolic study. Statistical significance was evaluated using JMP software v. 8.0 (SAS Institute, Cary, NC, USA). Significant differences between broomrape types were calculated according to the Tukey-Kramer honest significant difference (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 v. 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 used to infect the same host, tomato plants. The host plants were grown in pots containing the same number 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 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 (Figure 1A). The largest number and weight of parasitic plants were detected in *P. aegyptiaca* and *O. cernua*, while *O. cumana* had the lowest values (Figure 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 Profile Analysis of the Four Parasites

Global primary metabolic profile analyses were performed using 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 8 distinct groups: amino acids (19), sugars (11), sugar acids (6), polyols (5), TCA intermediates (4), organic acids (7), fatty acids (10), and others (3) (Supplementary Table S1). Similar results were also reported for *P. aegyptiaca* (Hacham et al. 2016; Kumar et al. 2022; Nativ et al. 2017) and *C. campestris* (Kumar and Amir 2021). A 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 (Figure 2A). The analysis showed clear separations between the *Phelipanche* and *Orobanche* genera on PC2 (Figure 2A). This indicates that in addition to morphological characteristics and phylogenetic data (Delavault 2015; Joel 2009), metabolic profiling can also be used to distinguish these two genera. A smaller separation was also seen between the two species of *Phelipanche*, *P. aegyptiaca* and *P. ramosa*, and between those belonging to *Orobanche*, *O. cernua* and *O. cumana*, as they were clearly separated by PC1 (Figure 2A). This suggests that each species has its own metabolic profile.

The PCA analysis suggests that the primary metabolic profiles of the parasite species differ. A PCA biplot was generated to determine which of the detected metabolites contributed to the



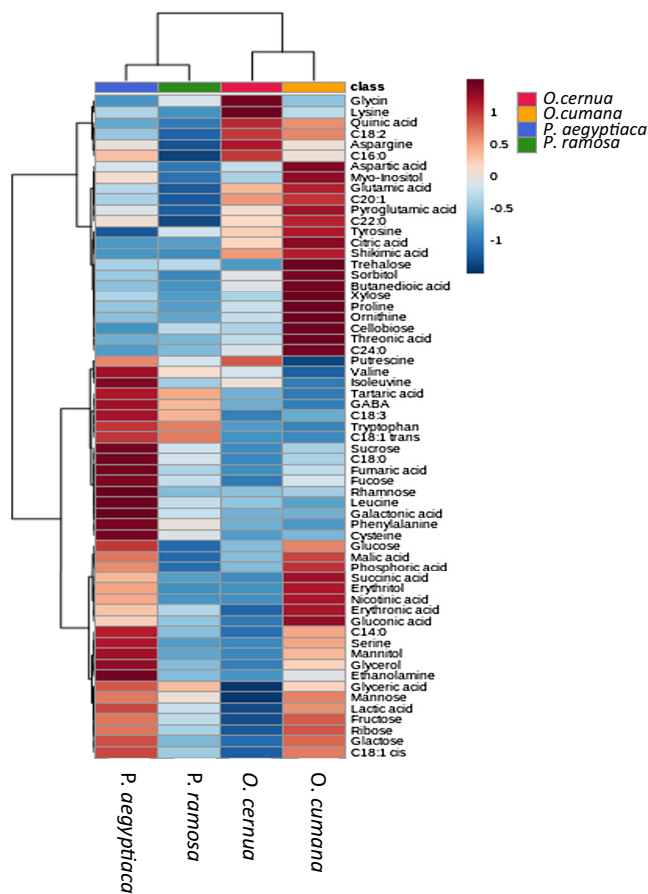
variance between the four broomrape species grown on tomato. Variables of metabolites such as gluconic acid, succinic acid, malic acid, nicotinic acid, erythronic acid, phosphoric acid, and glycine were highly associated with PC1. Therefore, they mostly contributed to the variance between *P. ramosa* and *P. aegyptiaca*, as well as between *O. cernua* and *O. cumana* (Figure 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 acids (C18:1, C18:2, C18:3 and C20:1) (Figure 2B). PC2 mainly separated between the two species of *Phelipanche* and of *Orobanche* (Figure 2B; Supplementary Figure S1).

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 10 different hosts (Kumar et al. 2022). In the latter case, sugars, phosphoric acid, shikimate, and polyols are the main factors influencing the variance in the parasite's metabolites (Kumar et al. 2022). This is also similar to the results of biplot analyses made from *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) (Figure 3). Compared with the other parasites, *P. ramosa* had the lowest contents of most of the metabolites detected (Figure 3).

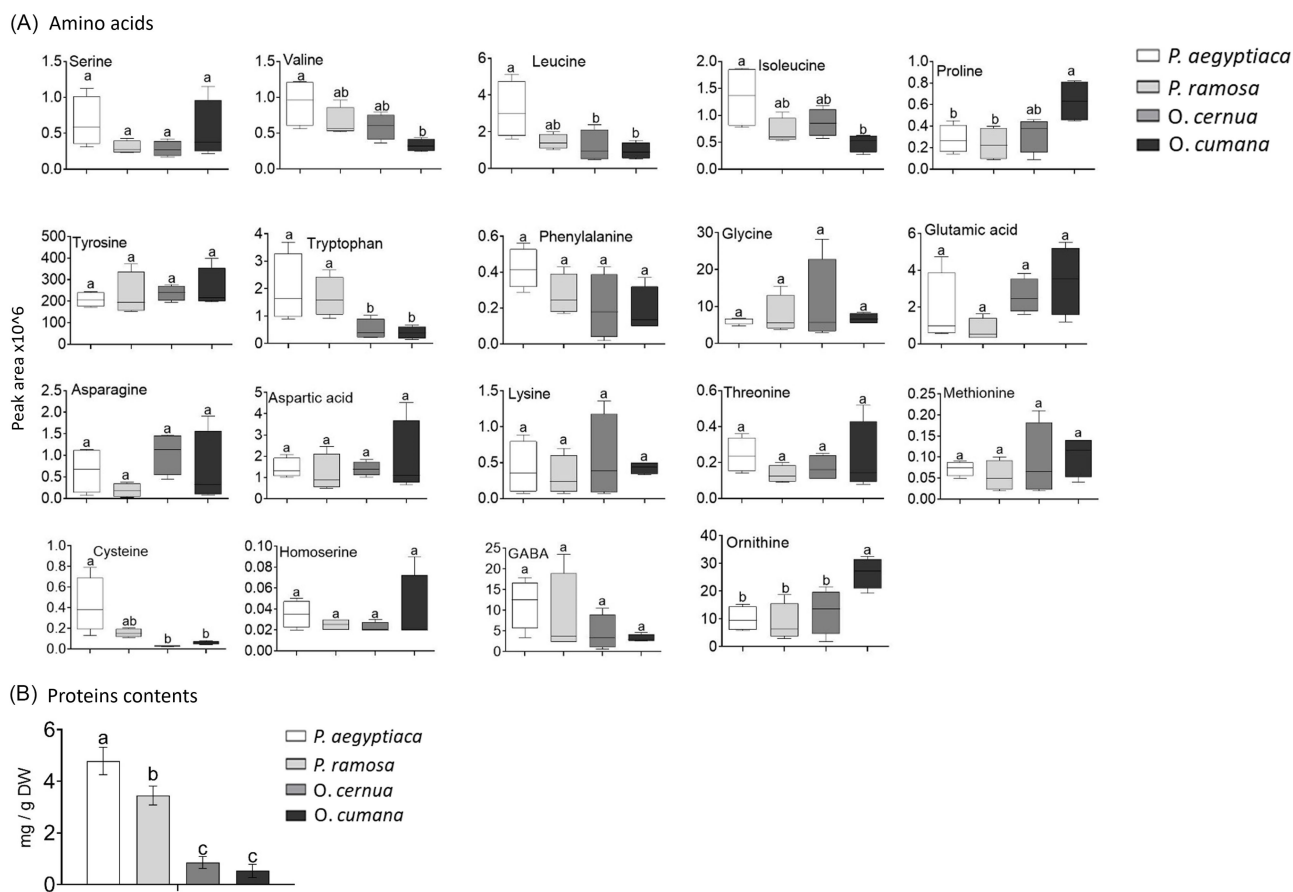
To obtain more information about the differences, each metabolite was analyzed separately. Among the 19 free amino acids that were detected, 12 did not differ significantly between the broomrape types (Figure 4). The levels of five amino acids were significantly higher in *P. aegyptiaca* compared with *O. cumana*, including the branched amino acids (valine, leucine, and isoleucine), tryptophan, and cysteine. Of these amino acids, only leucine, tryptophan, and cysteine had higher levels in *P. aegyptiaca* compared with *O. cernua*. The levels of the other two amino acids, proline and ornithine, were high in *O. cumana* compared with *P. aegyptiaca*, *P. ramosa*, and *O. cernua* (Figure 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 significantly lower protein content (Figure 4B). The results are in accordance with the finding that *P. aegyptiaca* has higher levels of five soluble amino acids compared with *O. cumana* (Figure 4A). A previous study revealed that *P. aegyptiaca* had a 9.8-fold higher protein content compared with roots of the tomato host (Hacham et al. 2016). Overall, the



**Figure 3.** Heat map of the 65 primary metabolites detected by gas chromatography–mass spectrometry (GC-MS). Distance is measured according to Pearson. The data represent four replicates for each broomrape type (*Phelipanche aegyptiaca*, *Phelipanche ramosa*, *Orobanche cernua*, and *Orobanche cumana*).

finding shows that two *Phelipanche* species (*P. aegyptiaca* and *P. ramosa*) had higher protein contents of about 3- to 5-fold compared with the *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 7 changed significantly between the broomrape types. *Orobanche cumana* had high levels of four sugars—mannose, trehalose, xylose, and cellobiose—compared with the other three broomrapes (Figure 5). *Phelipanche aegyptiaca* had a higher level of fucose than the other three broomrapes, while the levels of fructose, mannose, and ribose were higher than in *O. cernua*. *Phelipanche ramosa* had higher levels of mannose compared with *O. cernua*. 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 with *O. cernua*. *Phelipanche aegyptiaca* had lower levels of threonic acid and sorbitol and similar levels for the rest compared with *O. cumana*. *Phelipanche ramosa* had low levels of gluconic acid, threonic acid, myo-inositol, sorbitol and erythritol compared with *O. cumana* (Figure 5). The four TCA metabolites that were detected by GC-MS showed significant changes between the broomrape types. *Orobanche 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* (Figure 5). *Phelipanche ramosa* had lower levels of all four TCA



**Figure 4.** Levels of free amino acids and soluble proteins. (A) Free amino acids as detected by gas chromatography–mass spectrometry (GC-MS). Results are presented on a graph showing a compound level as the peak area ( $\times 10^6$ ) normalized to the internal standard (norleucine). (B) Total protein contents in the albumin fraction as measured using a Bradford assay. Data shown are means  $\pm$  SD of four replicates for each type of plant (*Phelipanche aegyptiaca*, *Phelipanche ramosa*, *Orobancha cernua*, and *Orobancha cumana*). Significance was calculated according to the Tukey-Kramer honest significant difference (HSD) test ( $P < 0.05$ ) and is identified by different lowercase letters.

metabolites. In comparison, *O. cernua* had lower levels of fumarate, malate, and succinate (Figure 5). Ten fatty acids were detected, seven of which showed significant differences between the four parasites (Figure 6). The rest of the metabolites exhibited a comparatively large variety among the four broomrape types. Shikimate and quinic acid were higher in *O. cumana* and *O. cernua* compared with *P. aegyptiaca* and *P. ramosa*. Butanedioic acid was higher in *O. cumana* (Figure 6).

Of the 65 metabolites identified, 26 did not change significantly between the four broomrapes species. They included 12 amino acids, 4 sugars, 2 sugar acids, 1 polyol, 2 organic acids, 3 fatty acids, and 2 others (Figures 4–6; Supplementary 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.

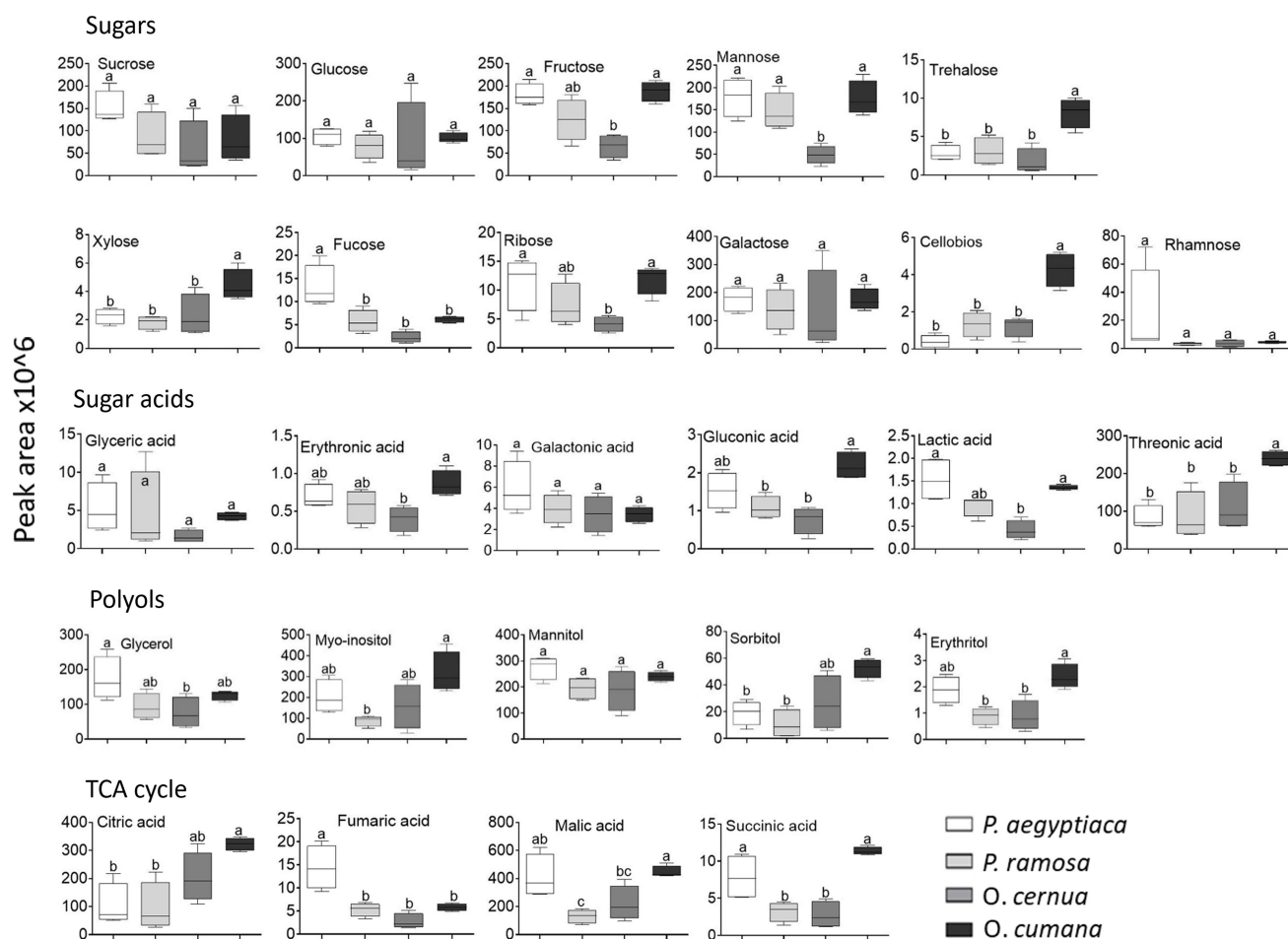
### Final Remarks

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 parasitic plants were grown on the same host.

The results show significant metabolic differences between two species of the two genera, *Orobancha* and *Phelipanche* (Figures 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, compared with *O. cernua*, *O. cumana* had significantly higher levels of sugars, sugar acid, polyols, TCA-organic acid, and several fatty acids (Figures 5 and 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

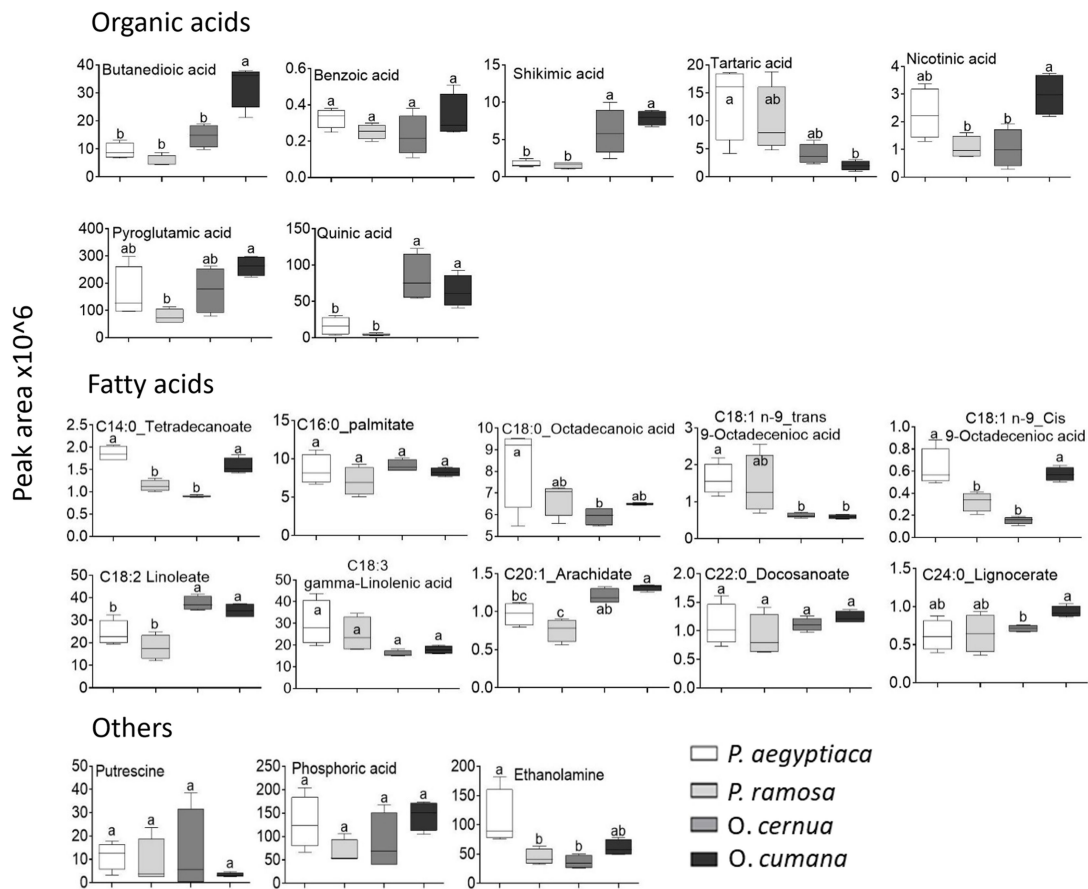


**Figure 5.** Levels of sugars, sugar acids, polyols, and tricarboxylic acid cycle (TCA) metabolites as detected by gas chromatography–mass spectrometry (GC-MS). Results are presented on a graph showing a compound level as the peak area ( $\times 10^6$ ) normalized to the internal standard (norleucine). Data shown are means  $\pm$  SD of four replicates for each type of plant (*Phelipanche aegyptiaca*, *Phelipanche ramosa*, *Orobanche cernua*, and *Orobanche cumana*). Significance was calculated according to the Tukey-Kramer honest significant difference (HSD) test ( $P < 0.05$ ) and is identified by different lowercase letters.

their metabolites, some of which were accumulated and others 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 carotenoid syntheses (e.g., Abbas 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 to a lesser extent 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 (Figure 5). Indeed, one of these enzymes was previously suggested to be a target to control *P. 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 could damage all 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 whether they are possible 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).



**Figure 6.** Levels of organic and fatty acids and three other metabolites as detected by gas chromatography–mass spectrometry (GC-MS). Results are presented on a graph showing a compound level as the peak area ( $\times 10^6$ ) normalized to the internal standard (norleucine). Data shown are means  $\pm$  SD of four replicates for each type of plant (*Phelipanche aegyptiaca*, *Phelipanche ramosa*, *Orobancha cernua*, and *Orobancha cumana*). Significance was calculated according to the Tukey-Kramer honest significant difference (HSD) test ( $P < 0.05$ ) and is identified by different lowercase letters.

**Supplementary material.** To view supplementary material for this article, please visit <https://doi.org/10.1017/wsc.2024.75>

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

**Funding statement.** 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.

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