Seed Pretreatment with Brassinolide Induces the Antioxidant Defense System of *Hellianthus annuus* against Sunflower Broomrape Infection

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Introduction of *Orobanche cumana*

Distribution of *O. cumana*

As a root holoparasitic angiosperm, sunflower broomrape (*Orobanche cumana* Wallr.) specifically affects sunflower (*Helianthus annuus* L.) crops in many countries like Spain, Russia, Turkey, and China.

Damages of *O. cumana*

The damage usually become **more and more severe** once *O. cumana* occurs. For example in Bayannuoer alone, one of the cites in Inner Mongolia, there have been **more than 13,500 ha** of planting areas subjected to infection of *O. cumana* currently, since the parasite was found in 2005.

Much more damage on confectionary sunflower than oil sunflower.

Control of *Orobanche cumana*

Chemical control: Herbicides

Agricultural control: Rotation

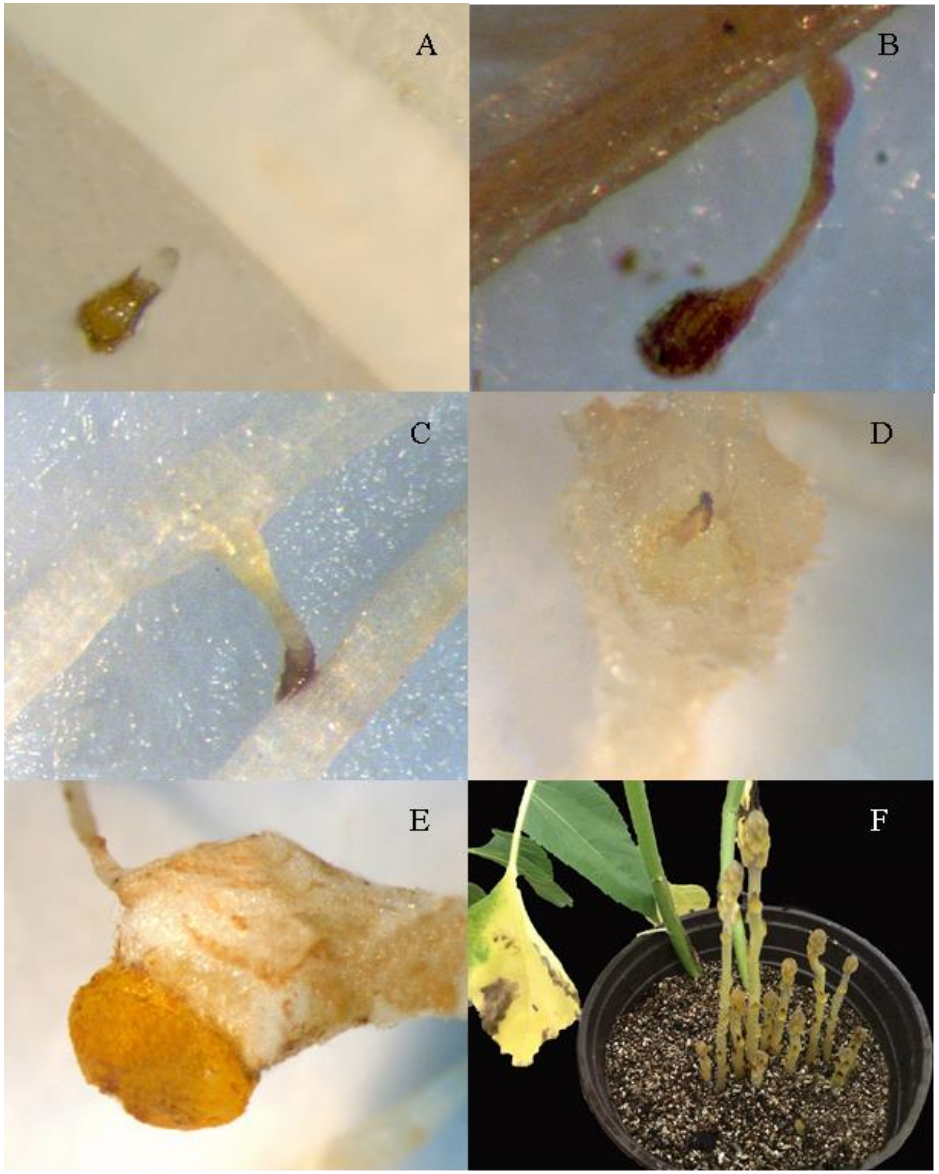
Biological control: *Fusarium* spp.

Manual control: Labor intensive

However, **the large seedbank of Broomrapes** in soil and **unpredictable infection** make the current control **methods not that effective**.

Genetic resistance in plants against *O. cumana* is still the most realistic, useful, economically and environmentally friendly strategy. Thus, it is very important to explore the **genetic resistance mechanisms** at a molecular level in sunflowers against the *O.cumana* infection for assistance in breading of resistant cultivars.

Development Stages of *Orobanche cumana*



We used **two contrasting cultivars** to compare their genetic differences.

(A) Germination (both *O. cumana* in JY207 and TK0409 can germinate),

(B) failed establishment on cv. JY207,

(C) attachment,

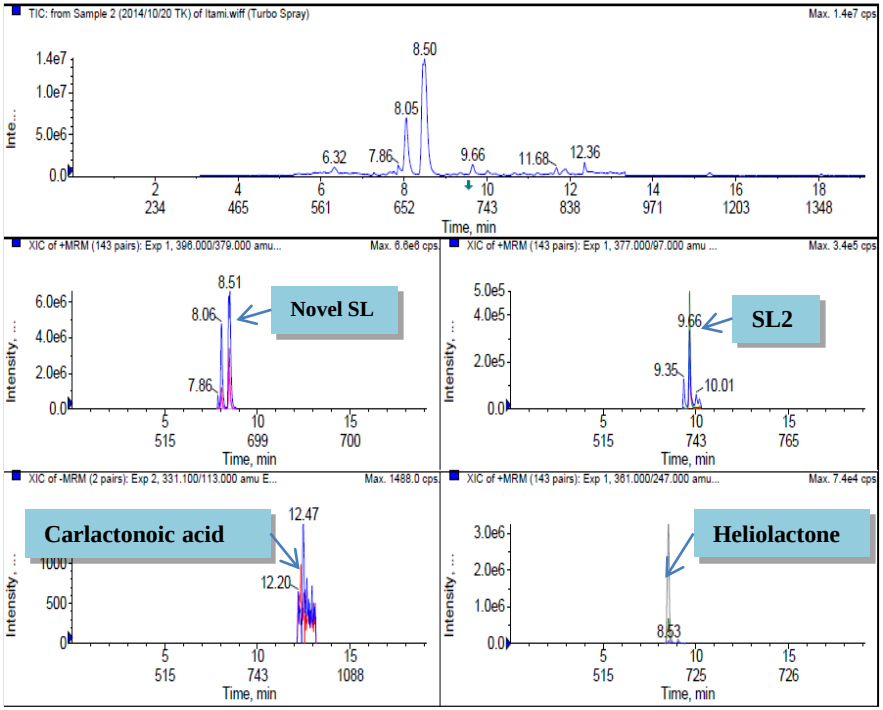
(D) tubercle development,

(E) underground shoot growth,

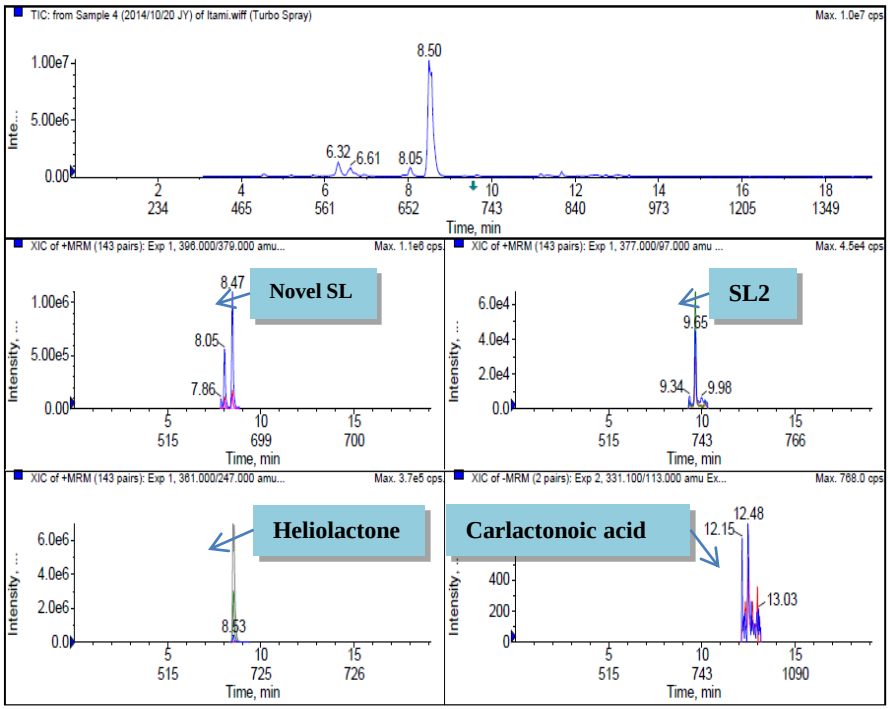
(F) shoot emergence on cv. TK0409.

Resistance Before Attachment

Research on resistance of host sunflowers against broomrapes can be roughly divided into two stages: **before attachment** and **after attachment**.
The former mainly involved in **germination stimulants.**



TK0409 (Susceptible cultivar)



JY207 (Resistant cultivar)

Four strigolactone-related compounds were detected in root exudates from both cultivars of sunflower. There were no significant differences in the germination stimulants

Effects of *O. cumana* and brassinolide (BR) on plant growth

Infection of *O.cumana*	BR concentration (mM)	Plant height (cm)	Fresh weight of seedling (g)	Dry weight of seedling (g)	Fresh weight of root (g)	Dry weight of root (g)	Fresh weight of *Orobanche* (g)	Dry weight of *Orobanche* (g)
No	0	44.83 f	22.61 c	2.14 bcd	4.38 c	0.30 c		
	0.0001	51.87 c	25.41 b	2.37 abc	5.41 ab	0.33 bc		
	0.01	59.30 a	29.19 a	2.83 a	6.14 a	0.39 a		
	1	54.17 b	27.76 a	2.51 ab	5.18 b	0.37 ab		
Yes	0	36.13 g	17.94 d	1.50 e	2.89 d	0.11 f	2.40 a	0.31 a
	0.0001	46.37 e	20.83 c	1.92 cde	3.06 d	0.16 ef	1.38 b	0.22 a
	0.01	53.23 b	25.29 b	1.94 cde	3.17 d	0.22 d	0.22 c	0.08 b
	1	50.37 d	21.69 c	1.72 ed	3.05 d	0.17 e	0.31 c	0.12 b

Effects of BR treatment (0, 0.0001, 0.01, 1 mM) and infection of *Orobanche cumana* on plant height, fresh weight and dry weight of sunflower cultivar TK0409, and effect of BR treatment on fresh weight and dry weight of established *O. cumana* on roots of sunflower plants.

0.01mM BR treatment significantly (P≤0.05) improved the biomass of *O. cumana*-infected sunflowers as compared with the respective untreated controls. By contrast, the biomass of *O. cumana* in terms of FW and DW was significantly (P≤0.05) decreased by the BR treatment as compared with the untreated controls.

Visual appearance of sunflower plants
from left to right: LO, L; MO, M; HO, H; CKO, LO; CKO, MO; CKO, HO
O, infection of *O. cumana;* CK, no BR treatment or infection of *O. cumana*
L, 0.0001 mM BR; M, 0.01 mM BR; H, 1 mM BR

Effects of *O. cumana* and BR on reactive oxygen species and malondialdehyde

		Leaves			Roots		
Infection of O.cumana	**BR concentration (mM)**	**MDA (nmol mg^{-1} protein)**	**OH^- (μmol g^{-1} FW)**	**H_2O_2 (μmol g^{-1} FW)**	**MDA (nmol mg^{-1} protein)**	**OH^- (μmol g^{-1} FW)**	**H_2O_2 (μmol g^{-1} FW)**
No	0	35.98 cd	66.8 c	186.97 ab	4.42 b	19.54 de	50.63 b
	0.0001	34.97 d	61.88 c	162.16 b	4.08 b	14.63 ef	47.46 b
	0.01	35.23 cd	58.27 c	126.40 c	0.91 d	12.54 f	37.31 c
	1	37.43 bcd	67.65 bc	169.75 b	1.15 cd	25.09 cd	49.44 b
Yes	0	41.73 a	100.54 a	210.58 a	6.3 a	49.75 a	72.77 a
	0.0001	38.38 bc	77.14 bc	178.71 b	4.24 b	30.96 c	52.67 b
	0.01	36.79 bcd	68.16 bc	127.01 c	1.32 cd	28.63 c	39.97 c
	1	39.46 ab	88.31 ab	208.14 a	1.73 c	38.17 b	65.74 a

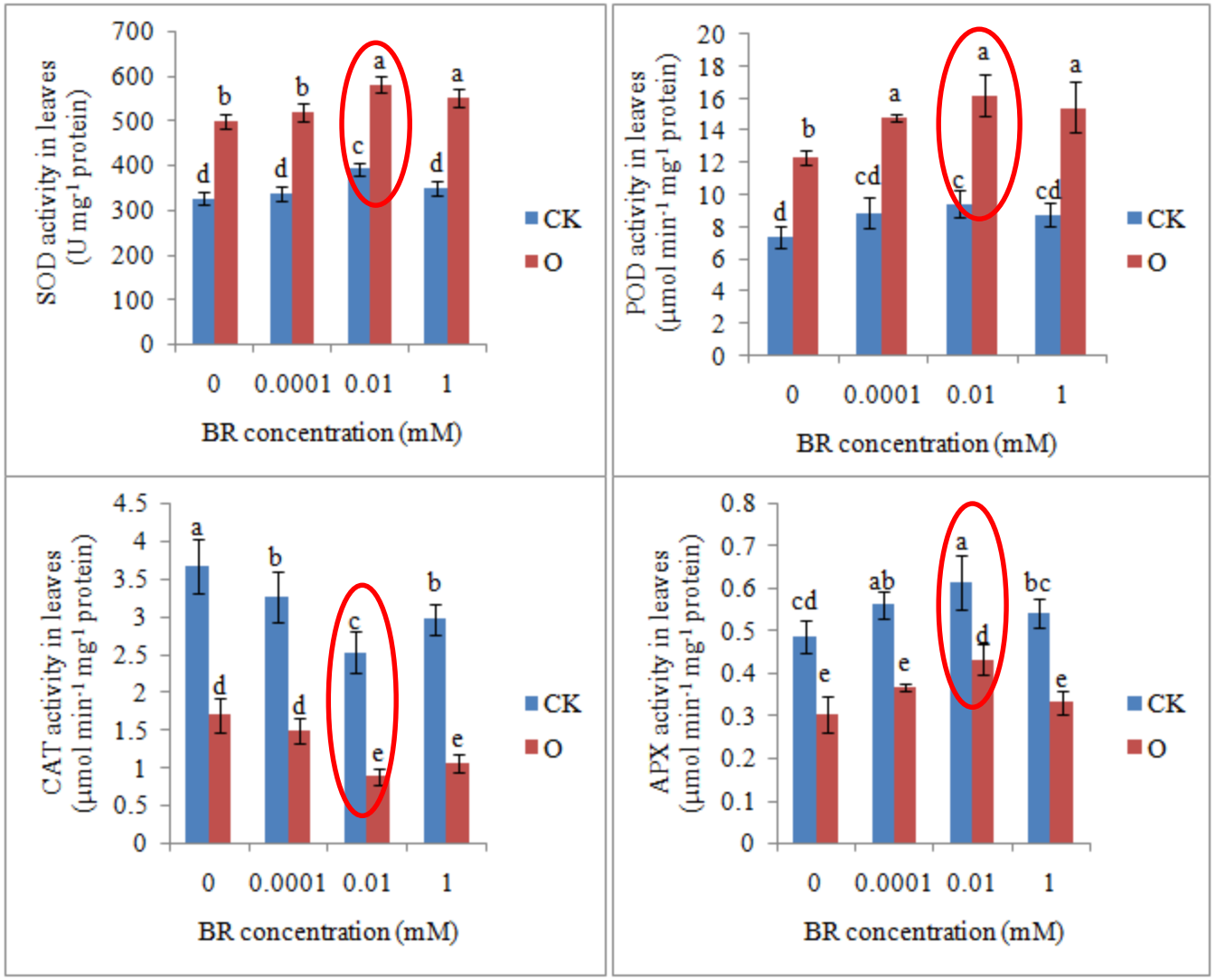
Effects of BR treatment and infection of *O.cumana* on malondialdehyde (MDA), hydroxyl ion (OH^-) and hydrogen peroxide (H_2O_2) contents in leaves and roots of sunflower cultivar TK0409.

BR treatment alone did not cause any significant (P≤0.05, LSD) changes in OH^- or MDA contents in leaves as compared with their respective controls. However, 0.01mM BR treatment alone significantly reduced the MDA ,OH^- and H_2O_2 contents in roots. *O. cumana* infection alone increased the contents of MDA, OH^-, H2O2, and by 16% 50.5%, 12.6% as compared with their respective controls. Contents of MDA, OH^-, H2O2 in the *O.cumana*-infected sunflower plants were significantly decreased by 0.01mM BR treatment by 11.8%, 32.2%, 39.7% in leaves and 79.0%, 21.12%, 45.1% in roots.

Effects of *O. cumana* inoculation on ROS level

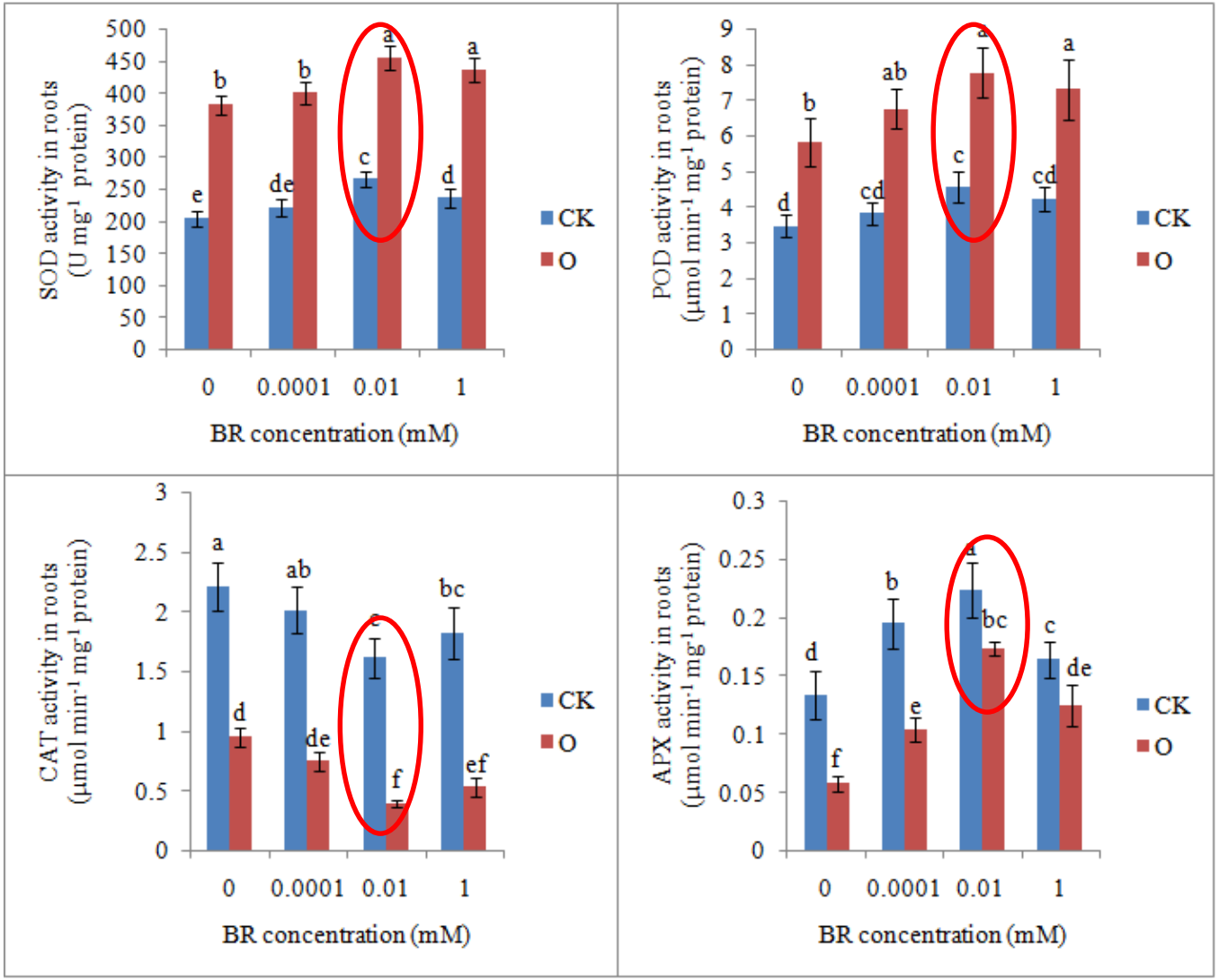
Accumulation of hydrogen peroxide H_2O_2 (A) and superoxide radical O_2^- (B) by DAB and NBT staining were observed in sunflower roots of susceptible cultivar TK0409

Activities of antioxidant enzymes in leaves



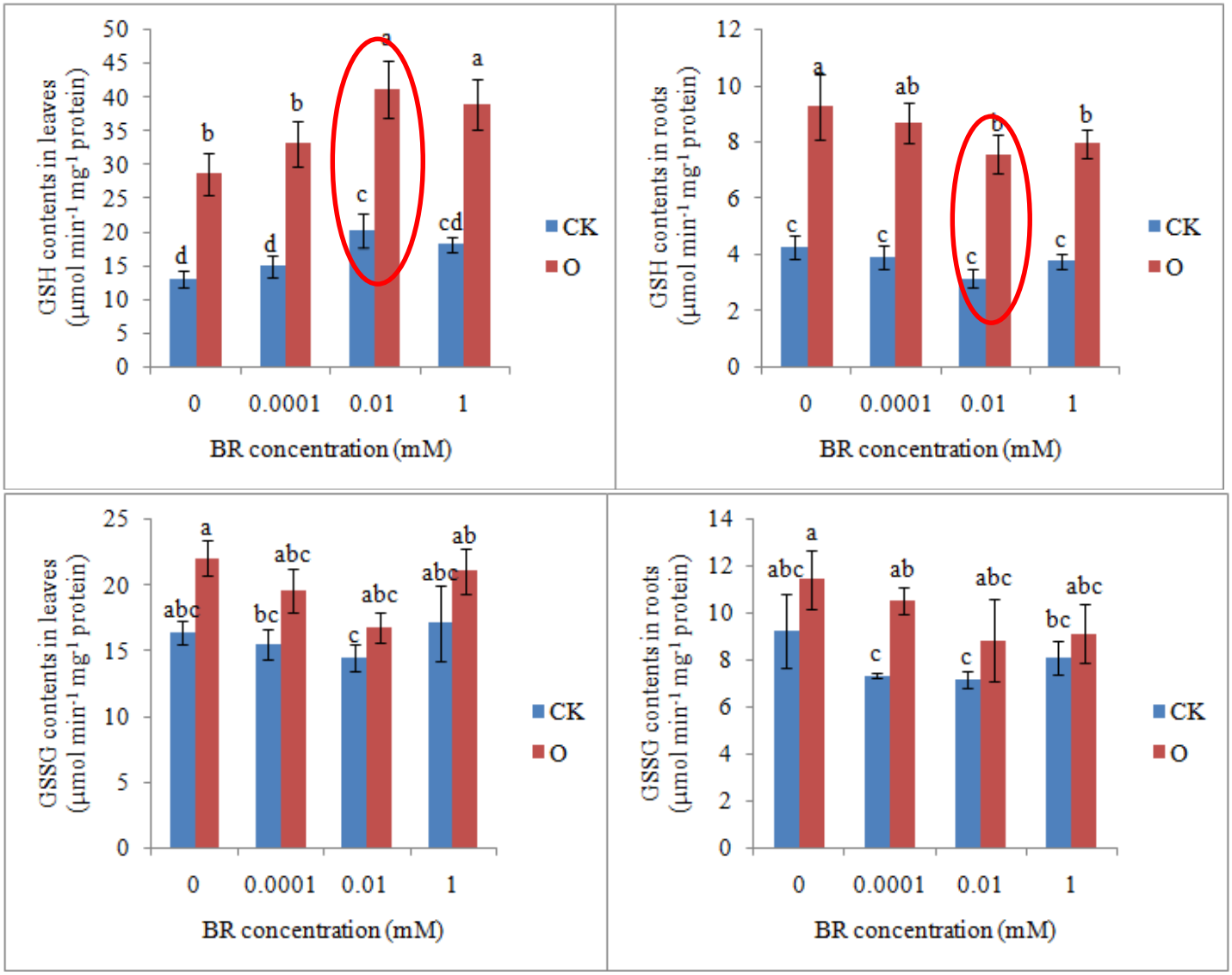
Effects of BR treatment and infection of *O.cumana* on the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD) in the leaves of sunflower cultivar TK0409 after 4 weeks of sowing. CK, sunflowers without inoculation, and O, sunflowers with inoculation.

Activities of antioxidant enzymes in roots



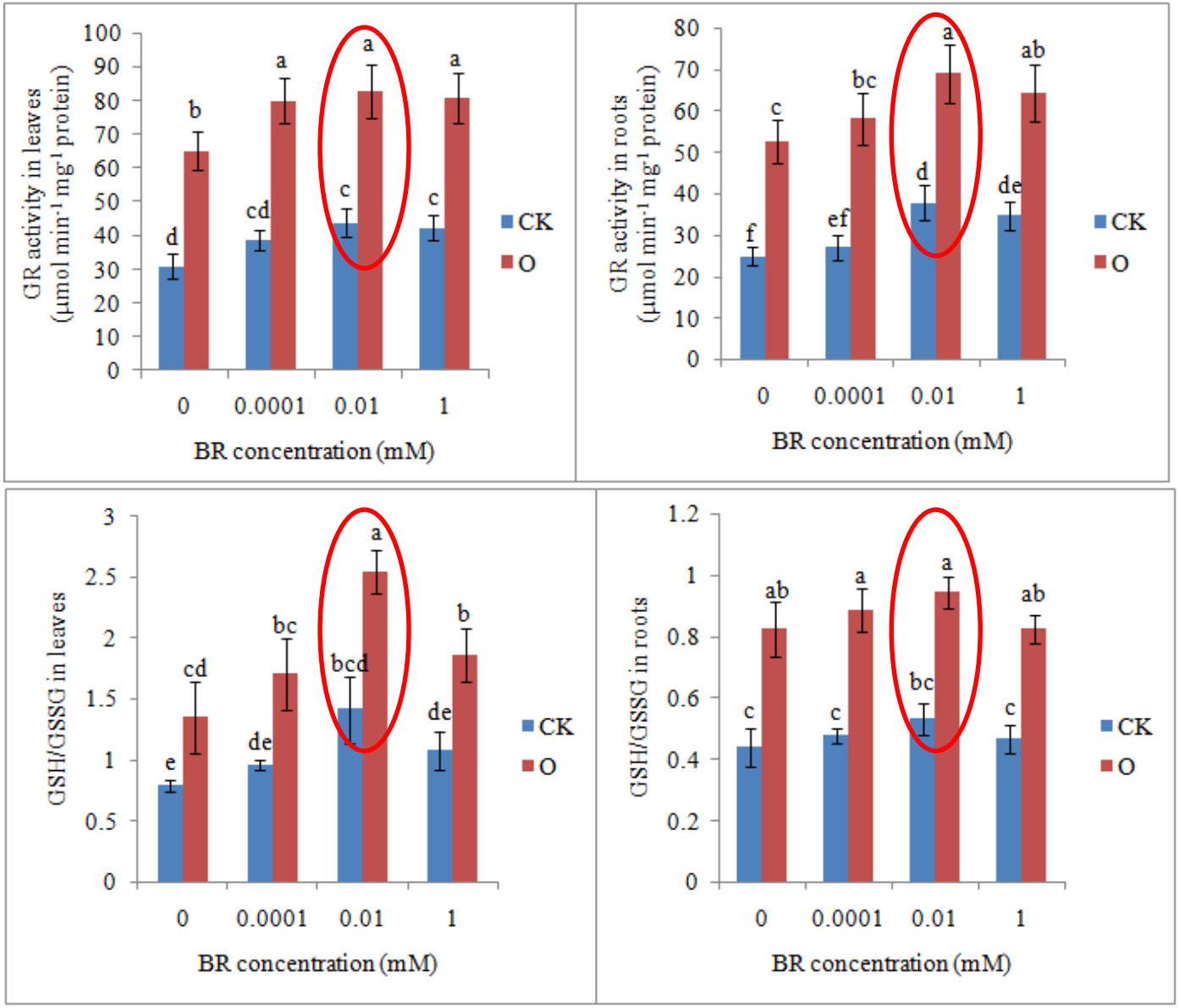
Effects of BR treatment and infection of *O.cumana* on the activities of SOD, APX, CAT and POD in the roots of sunflower cultivar TK0409 after 4 weeks of sowing.
CK, sunflowers without inoculation, and O, sunflowers with inoculation.

Changes in GSH and GSSG contents



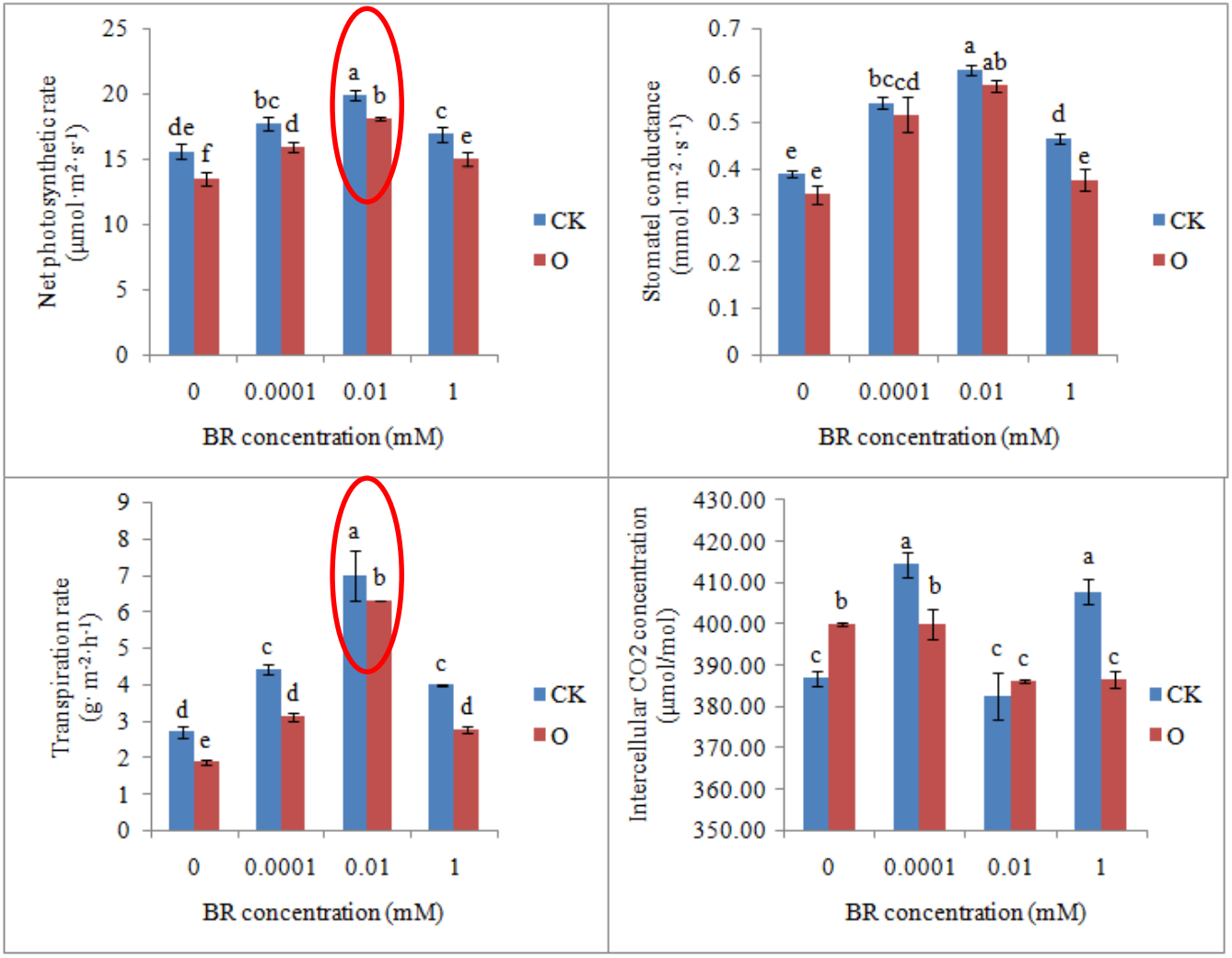
Effects of BR treatment and infection of *O.cumana* on the contents of reduced glutathione (GSH) , oxidised glutathione (GSSG) in leaves and roots of sunflower cultivar TK0409 after 4 weeks of sowing.

Effects of *O. cumana* and BR on GR activity and GSH/GSSG ratio



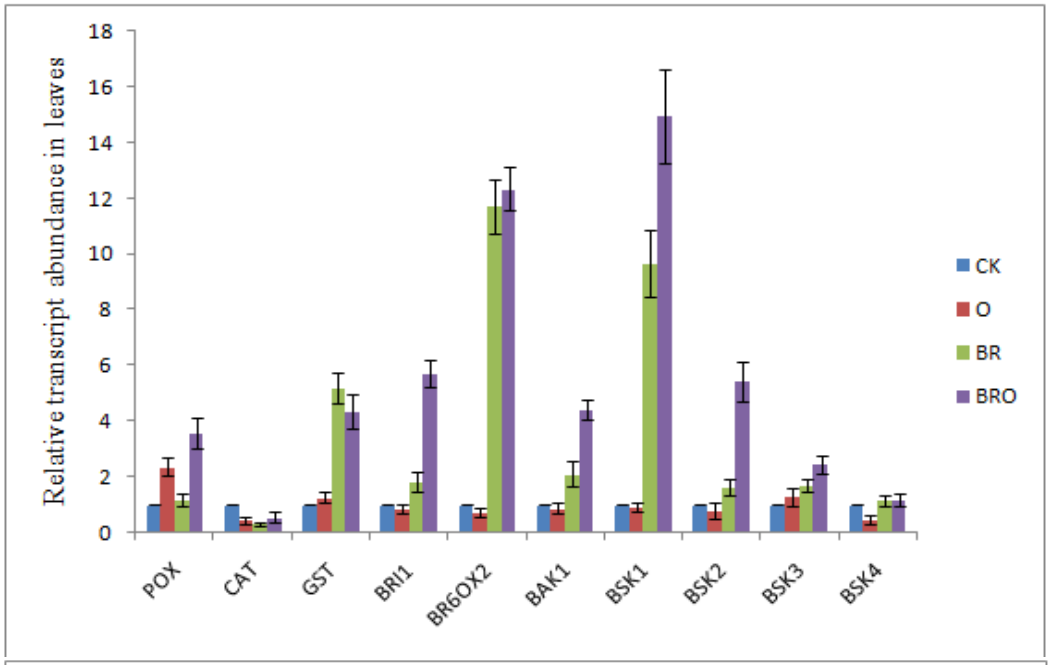
Effects of BR treatment and infection of *O.cumana* on the activity of glutathione reductase (GR) , GSH/GSSG ratio in leaves and roots of sunflower cultivar TK0409 after 4 weeks of sowing.

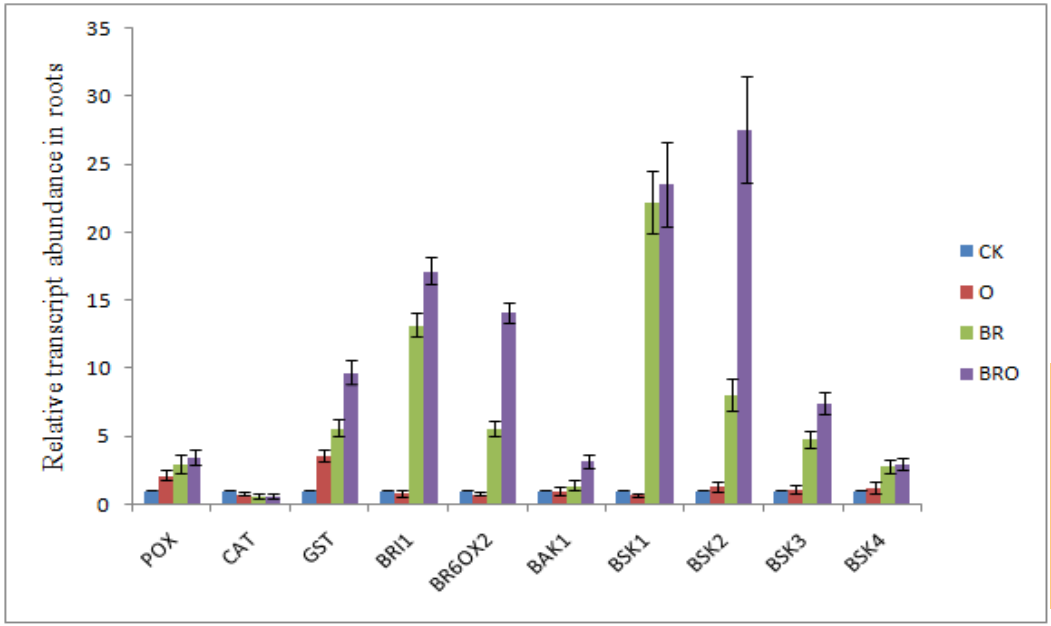
Effects of *O. cumana* and BR on plant photosynthesis



Effects of *O.cumana* on the net photosynthetic rate, stomatal conductance, intercellular CO2 concentration, transpiration rate of sunflower cultivar TK0409.

Effects of *O. cumana* and/or BR on expression of defence-related genes



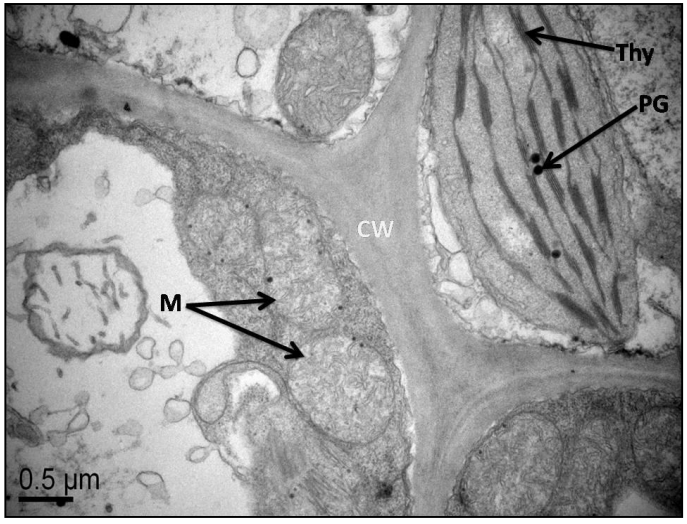


10 genes which are involved in encoding antioxidant enzymes, different metabolic pathways and resistance mechanisms were examined to analyze the effects of BR treatment on gene expressions in the *O.cumana*-infected sunflowers. Most of the genes such as pathogenesis-related (PR) genes, several antioxidant enzyme-related genes were up-regulated in response to the O. cumana infection, whereas CAT were down-regulated. The BR treatment significantly enhanced expression of most genes in both roots and roots of *O.cumana*-infected sunflower plants, whereas BSK4 were not affected by the BR treatment. In addition, BR treatment had a more significant affect on sunflower roots than leaves.

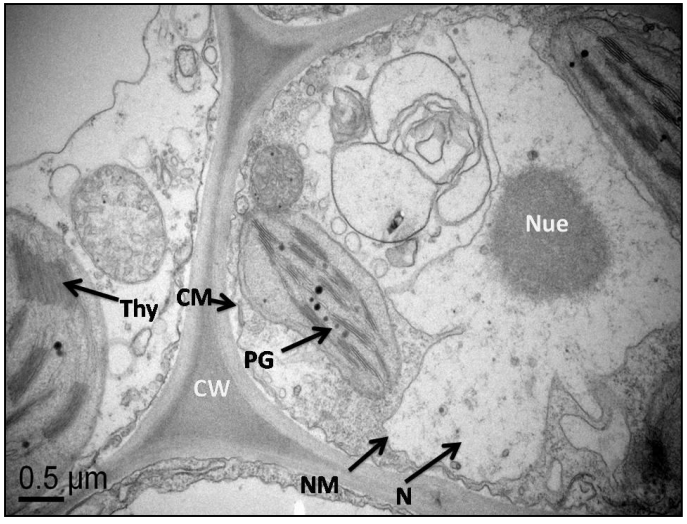
CK, no BR treatment or infection of *O. cumana*;
BR, treatment with 0.01 mM BR alone;
O, infection of *O. cumana* alone;
BRO, treatment with both 0.01 mM BR and infection of *O. cumana*.

Effects of *O. cumana* and BR on ultrastructural modifications

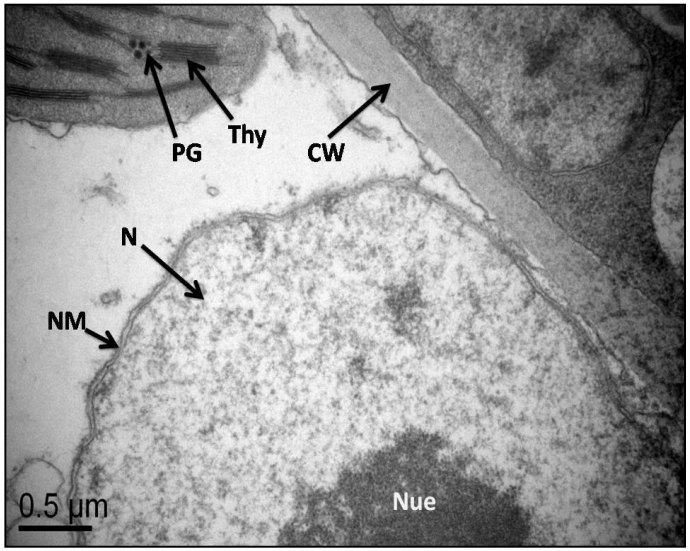
CK



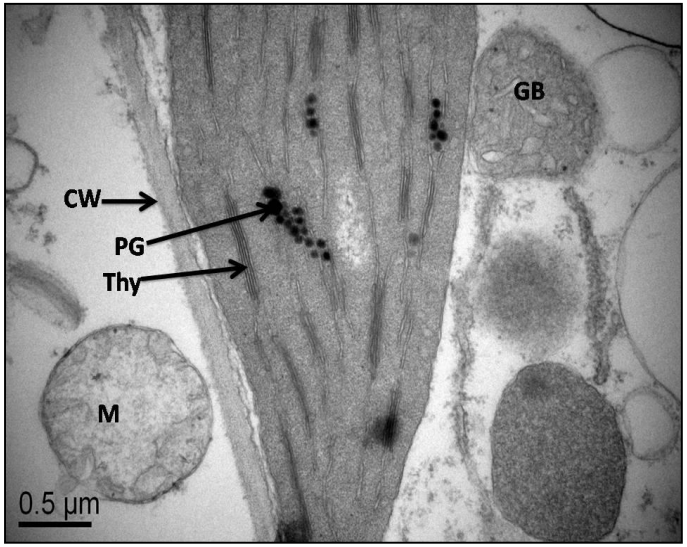
O



BR



BRO



Electron micrographs of leaf mesophyll of sunflowers. Compared with CK, O shows disturbed cell (CM), deshaped thylakoids (Thy), small-sized plastoglobuli (PG) and also damaged nucleus (N), nucleus membrane (NM) with disrupted nucleolus (Nue); BR shows well-developed nucleus (N) with nucleoli (Nue), nuclear membrane (NM), cell wall (CW). BRO shows well-developed cell wall (CW), mitochondria (M), golgi bodies (GB) and chloroplast structures.

Conclusions

1. **There is no significant difference in germination stimulants between two sunflower cultivars differing in resistance to *O. cumana* using current research methods;**

2. **ROS generation and its detoxification may act as signaling molecules that communicate downstream defense signals;**

3. **Defense proteins involved in successful recognition of invading parasites, accumulation of PR proteins might play an important role in resistance;**

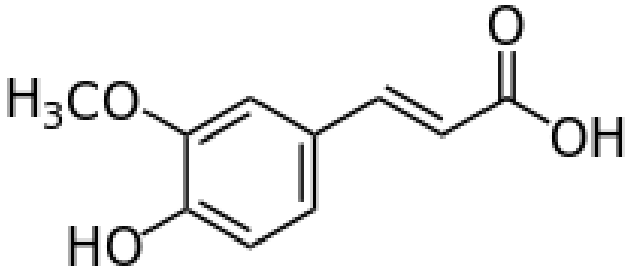
4. **Infection of *O. cumana* inhibits biosynthesis and signaling of plant growth regulators in host sunflowers.**

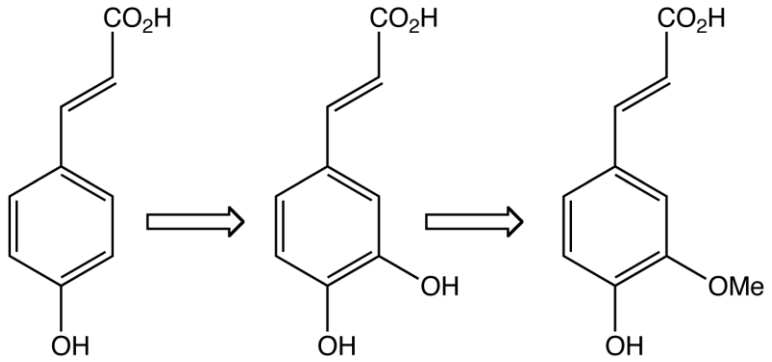
Related research work

1. Effective control on *Orobanche cumana* with Ferulic acid

Ferulic acid is a hydroxycinnamic acid, an abundant phenolic phytochemical found in plant cell walls, covalently bonded as side chains to molecules such as arabinoxylans. Together with **dihydroferulic acid**, it is a component of **lignocellulose**, serving to crosslink the lignin and polysaccharides, thereby conferring rigidity to the **cell walls**.

We treat the leaves of sunflower with Ferulic acid at early stage, finding that treated sunflower showed **better phenotype** (leaf chlorophyll, leaf area) and physiological parameter as peroxidase, catalase, etc. Compared with the control group, few or no *Orobanche cumana* tuberculars were attached on the root of sunflower. It suggested that Ferulic acid could help sunflower defense against *Orobanche cumana* invasion at the first stage. Further, we try to **understand the mechanism of Ferulic acid effect in the resistance system** and its regulatory pathway, finding related candidate genes and validation.

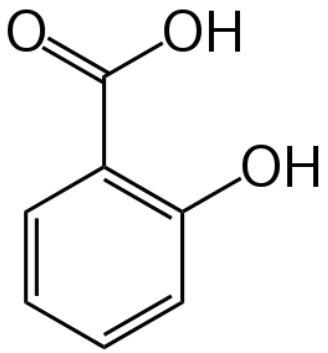




In plants, ferulic acid (right) is derived from phenylalanine, which is converted to 4-hydroxycinnamic acid (left) and then caffeic acid

2. Seed treatment with salicylic acid invokes defence mechanism

Salicylic acid (SA), a phenolic compound, plays a critical role in **plant defence responses against pathogens** (Halimet al., 2006). It is a **signaling hormone** which is involved in the induction of hypersensitive response (HR), expression of pathogenesis-related (PR) genes as well as the subsequent systemic acquired resistance (SAR) in plants.



Seed treatment with **1 mM Salicylic acid SA** increased *O. cumana*-susceptible cultivar of sunflower (TK0409) biomass in terms of plant height, fresh weight and dry weight by 10%, 13% and 26%, respectively, via **reducing the number and biomass of established *O. cumana***. The **enhanced expression of pathogenesis-related genes** (PR3 and PR12, encoding chitinase and defensin, respectively) after 4 weeks of inoculation indicated that **systemic acquired resistance was induced in the SA treated sunflower** in which the level of endogenous SA was also elevated in a dose-dependent manner. The **increased expression of a hypersensitive-responsive (HR) gene *hsr*** indicated that the **resistance of sunflowers might be associated with a hypersensitive reaction** which was activated by exogenous SA treatment.

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Thank you !