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Cross-resistance and biochemical mechanisms of resistance to indoxacarb in the diamondback moth, *Plutella xylostella*

Shuzhen Zhang [1], Xiaolei Zhang [1], Jun Shen, Dongyang Li, Hu Wan, Hong You *, Jianhong Li *

Hubei Insect Resources Utilization and Sustainable Pest Management Key Laboratory, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, PR China

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ABSTRACT

Indoxacarb belongs to a class of insecticides known as oxadiazines and is the first commercialized pyrazoline-type voltage-dependent sodium channel blocker. A moderate level of resistance to indoxacarb has evolved in field populations of *Plutella xylostella* from Central China. In the present study, cross-resistance, resistance stability and metabolic mechanisms of indoxacarb resistance were investigated in this moth species. A *P. xylostella* strain with a high level of resistance to indoxacarb was obtained through continuous selection in the laboratory. The strain showed cross-resistance to metaflumizone, beta-cypermethrin and chlorfenapyr, but no resistance to cyantraniliprole, chlorantraniliprole, abamectin, chlorfluazuron, spinosad and diafenthiuron compared with the susceptible strain. Synergism tests revealed that piperonyl butoxide (PBO) (synergistic ratio, SR = 7.8) and diethyl maleate (DEF) (SR = 3.5) had considerable synergistic effects on indoxacarb toxicity in the resistant strain (F_{58}). Enzyme activity data showed there was an approximate 5.8-fold different in glutathione *S*-transferase (GST) and a 6.8-fold different in cytochrome P450 monooxygenase between the resistant strain (F_{58}) and susceptible strain, suggesting that the increased activity of these two enzymes is likely the main detoxification mechanism responsible for the species' resistance to indoxacarb. These results will be helpful for insecticide resistance management strategies to delay the development of indoxacarb resistance in fields.

1. Introduction

The diamondback moth, *Plutella xylostella* (L.), is considered one of the most damaging insect pests of cruciferous vegetables around the world [1]. The annual *P. xylostella* associate losses and control costs in China are estimated at approximately US$ 0.77 billion [2]. Furthermore, a recent study showed that a conservative estimate of the annual global costs for this pest was approximately US$ 4–5 billions [3]. At present, the application of insecticides remains the principal strategy for *P. xylostella* management [2]. Due to the intensive and extensive application of insecticides for controlling *P. xylostella*, the species has evolved different levels of resistance to 95 active ingredients, including all major classes of insecticides, and has become one of the most resistant insect pests in the world [1,4–14]. The diamondback moth can evolve resistance to an insecticide after only two to three years following the introduction of the insecticide [1,5–8]. Currently, the number of insecticide resistance cases for *P. xylostella* is 777 as listed in the Arthropod Pesticide Resistance Database (APRD) [13]. Thus, insecticide resistance has been a major factor influencing *P. xylostella* control and management because the application of synthetic insecticides to crops remains the principal strategy for managing the species [2].

Indoxacarb, an oxadiazine insecticide, was discovered and developed by E.I. DuPont Co.; it was the first commercialized insecticide that blocked the sodium channels in neurons, and it gained approval for crop use for the first time in 1998 in Spain [14–16]. After several years of application, *P. xylostella* was the first species to develop resistance to indoxacarb in 2001 [8]. Currently, indoxacarb resistance in field populations of *P. xylostella* is at a medium to high level [10,17]. Synergism studies suggested that esterase, glutathione *S*-transferase (GST) and cytochrome P450-monooxygenase were involved in resistance to indoxacarb [1,7,18–20]. However, another study showed that two novel sodium channel mutations (F1845Y and V1848I) were associated with resistance to indoxacarb in field populations of the diamondback moth [17].

Cross-resistance is a common phenomenon in insects that can be conferred by metabolic mechanisms of resistance, such as elevated activities of esterase, GST and cytochrome P450 monooxygenase [1]. Knowledge of insecticide cross-resistance is helpful to delay the development of resistance by insecticide application tactics, such as insecticide mixtures and rotation of insecticides with different modes of action [20]. To date, cross-resistance to other insecticides has not been reported in indoxacarb resistant strains of *P. xylostella*.

* Corresponding authors.
E-mail addresses: youhong@mail.hazu.edu.cn (H. You), jianhl@mail.hzau.edu.cn (J. Li).
[1] These authors contributed equally to this paper.

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Studies of cross-resistance, resistance stability and metabolic detoxification mechanisms of laboratory-selected indoxacarb resistant strains are important strategies to delay indoxacarb resistance evolution in field populations of *P. xylostella*. In the present study, cross-resistance to metaflumizone, beta-cypermethrin, chlorfenapyr and other insecticides was determined in a laboratory-selected resistant strain of *P. xylostella* with a high level of indoxacarb resistance. Resistance stability was also studied in the resistant strain. Furthermore, the effects of three synergists on indoxacarb toxicity were tested to confirm the involvement of metabolic mechanisms in indoxacarb resistance in *P. xylostella*. Finally, metabolic enzymes were investigated in the resistant strain. The study provided important information on indoxacarb resistance characteristics that will be useful for the indoxacarb resistance management of *P. xylostella*.

2. Materials and methods

2.1. Susceptible and resistant strains

The susceptible strain of *P. xylostella*, obtained from Wuhan in 2002, has been maintained in the laboratory for >10 years without exposure to any insecticides. The resistant strain derived from the susceptible strain was selected with 1 to 500 mg/L of indoxacarb for 61 generations. The mortality during selection cycles was approximately 30–70%. Adults were fed with 10% honey solution and allowed to lay eggs on radish seedlings (*Raphanus sativus* L.). All populations were maintained at 25 ± 1 °C and 50–70% relative humidity with a 16:8 h light:dark photoperiod.

2.2. Insecticides

Six commonly used insecticides, including abamectin (2% Emulsifiable Concentrate (EC)), chlorfluazuron (5% EC), chlorfenapyr (10% EC), diafenthiuron (20% EC), beta-cypermethrin (20% EC) and chlorantraniliprole (5% EC), were provided by the Institute of Plant Protection of the Guangdong Academy of Agricultural Sciences, China. Cyantraniliprole (10% Suspension Concentrate (SC)) was supplied by E.I. DuPont Co. Spinosad (25 g/L SC) was supplied by the Dow AgroSciences Company. The indoxacarb (96%) and metaflumizone (97%) insecticides used in this study were technical grade compounds and were supplied by the Hubei Kangbaotai Fine-Chemicals Co., Ltd. Reagent grade triphenyl phosphate (TPP), diethyl maleate (DEM) and piperonyl butoxide (PBO) were purchased from Sigma.

2.3. Bioassay

Insecticide toxicity was assayed using the leaf-dipping method as described previously with a slight modification [21]. Young cabbage (*Brassica oleracea*) leaf discs (6.5 cm diameter) were cut from plants grown in a greenhouse without any insecticides. The technical grade compounds of indoxacarb and metaflumizone were dissolved in *N*, *N*-dimethylformamide, and then were serially diluted to five to seven required concentrations with distilled water containing 0.1% Triton X-100. Other insecticides were serially diluted to five to seven required concentrations with distilled water containing 0.1% Triton X-100. Three leaf discs were grouped together and dipped in solutions with different insecticide concentrations for 10 s. Control discs were only treated with 0.1% Triton X-100 solution and the control mortality was required to be <10%. All dipped leaf discs were dried at room temperature for approximately 120 min. The discs were then placed individually in plastic petri dishes (7.0 cm diameter). A total of 10 third-instar larvae were transferred to each dish, and four replicates were prepared and kept under standard conditions as previously described [10]. For the analysis of synergistic effects, 100 mg/L of DEM, TPP, and PBO were prepared. Larvae mortality was recorded after 48 h for abamectin, spinosad, chlorfenapyr, diafenthiuron, indoxacarb, and beta-cypermethrin, and after 72 h for chlorfluazuron, cyantraniliprole, and chlorantraniliprole. Larvae were considered dead if they could not be induced to move when touched with a brush.

2.4. Enzyme activity

Esterase activity was determined by the method of Asperen with modification [22]. The enzyme source was the supernatant of 10 third-instar larvae homogenized on ice in 1000 μL of precooled homogenization buffer (0.04 M sodium phosphate buffer, pH 7.0), then centrifuged at 4 °C and 10,000 × *g* for 15 min. To determine esterase activity, 1000 μL of preheated 0.3 mM α-NA and 200 μL of enzyme source (diluted 20–100-fold) were added to Eppendorf tubes; after 15 min, the dyeing reagent was added, and after 30 min, the optical density (OD) at 600 nm was recorded with an ultraviolet spectrophotometer.

GST activity using CDNB as substrates was measured as follows [23]. The enzyme source was prepared with 30 third-instar larvae homogenized in 1 mL of homogenization buffer (0.1 M sodium phosphate buffer with 1 mM EDTA, pH 6.5). The reaction solutions contained 50 μL of enzyme, 790 μL of the homogenization buffer, 30 μL of 30 mM CDNB and 30 μL of 30 mM GSH. An enzyme solution insteaded by buffer served as control. The OD at 340 nm was recorded at intervals of 1 s for 2 min at 25 °C with an ultraviolet spectrophotometer.

Cytochrome P450 monooxygenase activity was determined as follows [24]. The enzyme source was obtained from the homogenization of 50 third-instar larvae on ice in 1 mL of 0.1 M sodium phosphate buffer (pH 7.5) containing 1 mM of EDTA, 1 mM of DTT, 1 mM of PTU and 1 mM of PMSF, and centrifuged at 18,000 × *g* for 30 min. Afterwards, 600 μL of supernatant was added to an Eppendorf tube. The supernatant was recentrifuged at 18,000 × *g* for 30 min. The supernatant was then used as the substrate to test cytochrome P450 monooxygenase activity. Quantities of 750 μL of 2 μM PNA, 75 μL of 9.6 μM NADPH and 675 μL of enzyme solution were added to Eppendorf tubes. The mixed solutions were warmed in a water bath at 34 °C for 30 min, and the OD at 405 nm was recorded with an ultraviolet spectrophotometer.

The protein content of the enzyme solutions was determined by the Bradford method: 100 μL of sample, 700 μL of sterilized deionized water and 200 μL of protein assay dye reagent were added to Eppendorf tubes; after 5 min, the OD at 595 nm was recorded with an ultraviolet spectrophotometer.

2.5. Indoxacarb resistance stability in P. xylostella

Third-instar larvae from the resistant strain that had been maintained for seven generations in the absence of selection pressure, were bioassayed at F_{53}', F_{55}', F_{57}' and F_{58}'. The resistance ratio (RR) for the unselected populations was estimated by dividing their LC_{50} values by the LC_{50} value of the susceptible strain.

2.6. Statistical analysis

The mortality data were corrected by the control mortality using Abbott's formula; LC_{50} values, 95% confidence intervals and slopes were calculated by Probit analysis. The resistance ratio (RR) was calculated by dividing the LC_{50} value of the resistant strain by the LC_{50} value of the susceptible strain (Tables 1 and 4). The classification of the resistance levels followed Shao et al. [21]: RR ≤ 5-fold was classified as susceptible, RR = 5–10-fold as a low level of resistance, RR = 10–100-fold as a medium level of resistance and RR > 100-fold as a high level of resistance.

Table 1
Indoxacarb resistance changes in the laboratory population of *P. xylostella* with continuing selection.

Generation	No.	LC_{50} (95% CL) mg/L	Slope(SE)	χ^2 (df)	RR
Sus.	240	0.55 (0.35–0.83)	1.26 (0.27)	0.40 (3)	1.0
F_{31}	240	9.45 (6.51–17.48)	1.51 (0.31)	1.72 (3)	17.2
F_{32}	240	9.63 (4.97–65.32)	0.93 (0.27)	0.02 (2)	17.5
F_{33}	240	3.02 (0.91–5.34)	1.08 (0.29)	0.47 (2)	5.5
F_{36}	240	3.93 (2.36–5.86)	1.40 (0.30)	0.52 (3)	7.2
F_{38}	240	15.56 (10.18–32.22)	1.47 (0.27)	0.95 (2)	28.3
F_{43}	240	3.35 (2.27–5.18)	1.31 (0.27)	1.78 (2)	6.1
F_{44}	240	23.26 (12.36–90.76)	0.96 (0.22)	0.38 (3)	42.3
F_{45}	240	44.84 (23.30–205.07)	0.93 (0.23)	0.49 (3)	81.5
F_{47}	240	17.08 (12.17–24.95)	1.33 (0.21)	2.21 (2)	31.1
F_{48}	240	25.69 (16.11–52.67)	1.06 (0.22)	0.59 (2)	46.7
F_{50}	240	14.36 (9.55–20.56)	1.33 (0.22)	1.83 (3)	26.1
F_{51}	240	8.71 (4.67–13.39)	1.24 (0.21)	3.39 (3)	15.8
F_{53}	240	46.95 (35.07–62.42)	1.63 (0.19)	1.45 (3)	85.4
F_{54}	240	68.72 (47.09–104.97)	1.84 (0.23)	0.41 (3)	124.9
F_{55}	240	63.46 (44.86–88.01)	1.44 (0.21)	0.89 (2)	115.4
F_{57}	240	145.43 (105.65–219.74)	1.50 (0.22)	4.18 (2)	264.4
F_{58}	240	588.13 (312.64–2440.98)	1.00 (0.24)	0.85 (3)	1069.3
F_{60}	240	538.55 (298.76–1727.76)	0.87 (0.24)	0.94 (2)	979.2
F_{61}	240	574.12 (389.54–932.45)	1.19 (0.20)	2.21 (3)	1043.9

3. Results

3.1. Resistance development assessment

The resistant strain of *P. xylostella* (IR) was derived in the laboratory from a susceptible strain (IS) following 61 generations of continuous indoxacarb selection (Table 1). After 53 generations, the IR had evolved only an 85.4-fold resistance to indoxacarb. Afterwards, the RR increased quickly from the 54th to the 58th generation, reaching a 1069.3-fold increase by the 58th generation, and showed no further changes from the 58th to the 61st generation. Additionally, indoxacarb resistance changes in *P. xylostella* with cessation of selection were monitored when the RR was 85.4-fold. After cessation of selection, the RR showed a quick 12.5-fold decline from F_{53}' to F_{58}' (Table 2).

3.2. Cross-resistance

Compared with the IS, the IR showed cross-resistance to three insecticides, including beta-cypermethrin (RR = 8.7), metaflumizone (RR = 20.9), and chlorfenapyr (RR = 8.3). No significant cross-resistance to other insecticides was observed (Table 3).

3.3. Synergistic effects

The synergistic effects of PBO, TPP, and DEM with indoxacarb against the IR and the IS are shown in Table 4. Significant synergisms were found for PBO and DEM in the IR (F_{58}) (synergistic ratio, SR = 7.8- and 3.5-fold, respectively); no synergy was observed for TPP in either strain.

Table 2
Indoxacarb resistance changes in the laboratory population of *P. xylostella* with cessation of selection.

Generation	No.	LC_{50} (95% CL) mg/L	Slope (SE)	χ^2 (df)	RR
F_{53}'	240	46.95 (35.07–62.42)	1.63 (0.19)	1.45 (3)	85.4
F_{55}'	240	31.72 (22.41–45.09)	1.63 (0.29)	0.80 (2)	57.7
F_{57}'	240	13.33 (9.61–18.08)	1.87 (0.37)	0.28 (2)	24.2
F_{58}'	240	6.85 (5.45–9.07)	2.42 (0.34)	3.55 (3)	12.5

Table 3
Cross-resistance spectrum in laboratory indoxacarb-resistant strains of *P. xylostella*.

Insecticides	Strains	LC_{50} (95% CL) mg/L	Slope (SE)	χ^2 (df)	RR
Beta-cypermethrin	S	5.41 (3.80–19.67)	1.88 (0.58)	3.13 (2)	
	F_{53}	47.28 (28.54–97.17)	1.08 (0.26)	0.51 (3)	8.7
Metaflumizone	S	1.24 (0.92–1.62)	2.16 (0.40)	1.51 (3)	
	F_{53}	25.90 (14.57–52.48)	1.12 (0.26)	0.89 (3)	20.9
Diafenthiuron	S	20.83 (15.28–27.19)	1.85 (0.24)	1.60 (2)	
	F_{57}	25.47 (17.12–34.13)	1.84 (0.30)	3.16 (2)	1.2
Chlorfenapyr	S	0.36 (0.24–0.57)	1.38 (0.29)	2.70 (3)	
	F_{58}	2.99 (2.51–3.71)	3.87 (0.59)	1.26 (3)	8.3
Spinosad	S	0.54 (0.36–1.00)	1.50 (0.27)	2.78 (3)	
	F_{58}	0.86 (0.62–1.52)	2.07 (0.39)	2.00 (3)	1.6
Chlorantraniliprole	S	0.22 (0.16–0.31)	1.68 (0.23)	1.23 (2)	
	F_{58}	0.21 (0.14–0.34)	1.47 (0.30)	0.08 (3)	1.0
Chlorfluazuron	S	1.04 (0.45–370.12)	1.21 (0.49)	0.08 (3)	
	F_{58}	1.00 (0.70–1.83)	1.63 (0.32)	3.53 (2)	1.0
Abamectin	S	0.05 (0.04–0.07)	1.45 (0.21)	0.41 (3)	
	F_{58}	0.04 (0.03–0.08)	2.29 (0.43)	0.49 (2)	0.8
Cyantraniliprole	S	0.20 (0.15–0.28)	2.39 (0.29)	0.72 (3)	
	F_{58}	0.19 (0.14–0.26)	1.98 (0.32)	3.63 (3)	1.0

3.4. Metabolic enzyme activity

Esterase activity of the IR (F_{58}) was 1.47 μmol/min/mg protein, with a 1.6-fold increase over that of the IS (0.93 μmol/min/mg protein) (Table 5). Cytochrome P450 monooxygenase activity using p-nitroanisole as substrate in the IS and IR is shown in Table 5. The cytochrome P450 monooxygenase activity of the IR (F_{58}) was 6.8-fold compared with that of the IS. GST activity of the IR (F_{58}) was 5.8-fold as that of the IS.

4. Discussion

Indoxacarb belongs to a relatively new class of sodium channel blocker insecticides [17]. Due to the intensive use of indoxacarb, it had been reported that resistance had occurred in field populations of some insect species, such as *Aedes albopictus*, *Choristoneura rosaceana*, *Earias vittella*, *Helicoverpa armigera*, *Heliothis virescens*, *Lobesia botrana*, *Musca domestica*, *Sitophilus zeamais*, *Spodoptera exigua*, *Kampimodromus aberrant*, *Spodoptera litura*, *Tuta absoluta* and *P. xylostella* [10,13,17,18,25–35]. Currently, a high level of resistance to indoxacarb in *P. xylostella* is common in South China [17], whereas only low and moderate levels of resistance are found in Central China [10]. Thus, to delay indoxacarb resistance evolution in field populations of *P. xylostella*, studies concerning resistance stability, cross-resistance pattern as well as resistance mechanisms in this species are very important to develop sustainable pest management strategies in the field.

Previous studies have shown that *P. xylostella* can maintain resistance to indoxacarb under selection pressure in the laboratory [7]. Continuous selection resulted in a high level of resistance from an 813-fold resistance in the 1st generation to a 2594-fold resistance by the 8th generation [7]. This resistance was unstable and disappeared after a few generations without insecticide pressure [7]. Similarly, in the present study, *P. xylostella* selected with indoxacarb for 58 generations in the laboratory developed a 1069.3-fold resistance. The resistance was also unstable and disappeared after a few generations without insecticide pressure. This instability might be associated with an incompletely recessive trait of indoxacarb resistance and fitness costs [36]. An unstable resistance is useful to control insecticide resistance by strategically stopping the use of an insecticide to prolong its efficiency.

Cross-resistance between indoxacarb and other insecticides has been reported in *P. xylostella* strains in several studies [1,37]. Some reports indicated that cross-resistance could result from metabolic detoxification enzymes (cytochrome P450 monooxygenase, GST and esterase), mutation at an insecticidal target site or delayed cuticular penetration [38]. In the present study, there was cross-resistance between indoxacarb and metaflumizone in the IR. Metaflumizone belongs

Table 4
Synergism of TPP, DEM and PBO on indoxacarb in different strains of *P. xylostella*.

Generation	Insecticides	LC_{50} (95% CL) mg/L	Slope (SE)	χ^2 (df)	SR
Unselected	Indoxacarb	0.72 (0.51–0.95)	1.87 (0.30)	0.17 (3)	1.0
	Indoxacarb + PBO	1.50 (1.05–2.50)	1.52 (0.24)	0.31 (2)	0.5
	Indoxacarb + DEM	0.92 (0.70–1.20)	12.7 (0.45)	0.33 (2)	0.8
	Indoxacarb + TPP	0.72 (0.56–1.00)	2.09 (0.32)	0.68 (3)	1.0
F_{47}	Indoxacarb	17.08 (12.17–24.95)	1.33 (0.21)	2.21 (2)	1.0
	Indoxacarb + PBO	15.32 (11.14–23.89)	1.58 (0.29)	0.99 (2)	1.1
	Indoxacarb + DEM	18.16 (12.38–28.76)	1.53 (0.25)	0.17 (2)	0.9
	Indoxacarb + TPP	11.60 (7.51–18.12)	1.56 (0.23)	1.86 (3)	1.5
F_{58}	Indoxacarb	588.13 (312.64–2440.98)	1.00 (0.24)	0.85 (3)	1.0
	Indoxacarb + PBO	75.73 (45.62–129.11)	1.17 (0.24)	1.49 (3)	7.8
	Indoxacarb + DEM	170.68 (125.90–268.40)	1.92 (0.34)	1.33 (3)	3.5
	Indoxacarb + TPP	400.16 (225.83–2049.03)	1.80 (0.48)	0.41 (3)	1.5

to a new chemical class of insecticides with the same mode of action as indoxacarb, a sodium channel blocker in the central nervous system [39–41]. A more recent study also showed that field populations of *P. xylostella* with a high level of resistance to indoxacarb also showed resistance to metaflumizone and that sodium channel mutations may result in resistance against the two insecticides in this species [17]. These results suggest that cross-resistance between the two insecticides in *P. xylostella* can be caused by sodium channel mutations. Furthermore, beta-cypermethrin is a pyrethroid insecticide and chlorfenapyr is a pyrrole insecticide. These two insecticides bind at different target sites, implying that metabolic detoxification might be responsible for cross-resistance between them and indoxacarb.

P. xylostella and other insect pests have evolved mechanisms of insecticide resistance to detoxify or reduce their sensitivity to insecticides [1,5,12,42,43]. Increased detoxification can occur by gene duplication of carboxylesterase and increased transcription of cytochrome P450 monooxygenase and GST [42,44]. Moreover, point mutations in the target site can also reduce insecticide binding [5,42]. However, in many cases, up-regulation of the detoxifying enzymes is the most common mechanism of insecticide resistance [42]. Many studies have shown that esterase, GST and cytochrome P450 monooxygenase play an important role in the detoxifying strategies of insect pests [42,44–48]. Currently, indoxacarb resistance through metabolic detoxification has been reported in *P. xylostella* and other insect pests [7,18,19,49]. For example, a synergism study suggested that indoxacarb resistance in *P. xylostella* was esterase mediated [7]. In another study, GST and esterase were involved in resistance to indoxacarb [49]. It was also reported that indoxacarb resistance in *P. xylostella* was associated with cytochrome P450 monooxygenase but not with esterase or GST [18]. However, in the present study, enhanced activity of GST and cytochrome P450 monooxygenase is likely the main detoxification mechanism responsible for resistance by biochemical analysis and synergistic suppression (PBO and DEM).

This study demonstrates that *P. xylostella* can develop resistance to indoxacarb and that enhanced detoxification by GST and cytochrome P450 monooxygenase is mainly responsible for this resistance. The study represents a first step towards understanding the biochemical mechanisms of indoxacarb resistance in selected strains in our laboratory. The target insensitivity mechanism accompanying the appearance of a higher resistance level will be the focus of further investigation. The inheritance patterns of indoxacarb resistance in *P. xylostella* will also require additional research to confirm the resistance mechanism and to establish more efficient management strategies for this pest.

Table 5
Mean (±standard error) metabolic enzyme activity in populations of *P. xylostella*.

Strains	Esterase μmol/mg pro/min	GST μmol/mg pro/min	Cytochrome P450 monooxygenase nmol/mg pro/min
Sus.	0.93 ± 0.03b	0.73 ± 0.01c	0.16 ± 0.002b
F_{47}	0.76 ± 0.03b	0.86 ± 0.01b	0.20 ± 0.005b
F_{58}	1.47 ± 0.54a	4.25 ± 0.24a	1.09 ± 0.014a

Competing interests

The authors have declared that no competing interests exist.

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