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Responses of seeds of typical *Brassica* crops to tetracycline stress: Sensitivity difference and source analysis

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ABSTRACT

Antibiotics can induce adverse effects on plants. *Brassica* crop seeds, for their advantages, are used widely in seed germination test to investigate phytotoxicity of substances. However, their performances on evaluating antibiotics remain to be studied to select sensitive species for control of potential risks. In this work, common species of Chinese cabbage (*Brassica rapa* L.), edible rape (*Brassica napus* L.), and cabbage (*Brassica oleracea* L.) with three cultivars each were selected to compare and analyze the sensitivity difference of their seeds to tetracycline (TC) stress. Results showed that the ratio of axis to cotyledon (RAC) by fresh weight was an alternative endpoint besides radicle length (RL) in the test. The species sensitivity distribution (SSD) based on the effective concentrations causing x% inhibition (EC_x) in RL of seeds exposed to TC was applied to compare the sensitivity of seeds and estimate the hazardous concentration for x% species (HC_x). From the species-dependent sensitivity and the sensitivity difference of cultivars in the same species of seeds to TC, the performance of Chinese cabbage was the best in the study. The sensitivity of seeds to TC could be evaluated by EC_{20} related to seed physical traits and germination indices, while the extent of seeds affected by TC could be evaluated by EC_{50} related to the composition of seed storage reserves. We recommended that it was a new idea to analyze responses of different seeds to TC at large scale according to seed innate characteristics.

1. Introduction

Antibiotics have been heavily used in pharmaceuticals and animal feedstuffs, which results environmental health risks causing worldwide concerns, such as the spread of antibiotic-resistance genes (Huijbers et al., 2015) and the toxicity of antibiotics to nontarget organisms (Agathokleous et al., 2018; Brox et al., 2016). In 2015, the Center for Disease Dynamics, Economics & Policy of World Health Organization published a report on the status of the world's antibiotics to push countries in different regions to make effective efforts in reducing the environmental health risks caused by the use of antibiotics (CDDEP, 2015). Antibiotics enter the environment through wastewater and biosolid from human and animal excreta as well as manufacturing waste (Munir et al., 2011; Williams-Nguyen et al., 2016). The persistent entry of antibiotics into environment makes them pseudo-persistent contaminants (Kalaji and Rastogi, 2017) with the levels of ng L^{-1} − μg L^{-1} in water and μg kg^{-1} − mg kg^{-1} in soil (Zhang et al., 2015; Li et al., 2016). Antibiotics can lead to adverse effects on nontarget organisms at environmentally relevant levels, such as plants (Hillis et al., 2011; Minden et al., 2017).

Seed germination and post-germination test (hereinafter referred to as seed germination test) is an effective and efficient method for testing the phytotoxicity of single substances or mixtures, such as wastewater (Salian et al., 2018; Xing et al., 2019), compost (Hase and Kawamura, 2012; Luo et al., 2018), biochar (Ghidotti et al., 2017; Liang et al., 2019; Visioli et al., 2016), petroleum hydrocarbons (Kaur et al., 2017; Wang et al., 2001), nanomaterials (Lin and Xing, 2007; Song et al., 2017), and heavy metals (Di Salvatore et al., 2008; Li et al., 2005) as

Abbreviations: m_{100}, 100-seed weight; SS, seed size; TS, total sugar; TP, total protein; TL, total lipid; IGT, initial germination time; RL, radicle length; GE, germination energy; RER, radicle elongation rate; RAC, ratio of axis to cotyledon by fresh weight; ZS5, *Brassica rapa* L. cv. Zaoshu No. 5; JQ3, *Brassica rapa* L. cv. Jingqiu No. 3; GLQZ, *Brassica rapa* L. cv. Gailiang Qingza; ZYZ19, *Brassica napus* L. cv. Zhongyouza No. 19; ZY50, *Brassica napus* L. cv. Zheyou No. 50; ZY51, *Brassica napus* L. cv. Zheyou No. 51; JF1, *Brassica oleracea* L. cv. Jingfeng No. 1; XZG11, *Brassica oleracea* L. cv. Xin Zhonggan No. 11; ZG15, *Brassica oleracea* L. cv. Zhonggan No. 15

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well as antibiotics (Bellino et al., 2018; Luo et al., 2019; Pan and Chu, 2016). Crop seeds, from the plants most closely related to human beings, are the commonly used species in seed germination test to evaluate the phytotoxicity of pollutants, mainly because their habits are well known and their germination conditions can be easily satisfied in a laboratory (Wang and Freemark, 1995). Interestingly, *Brassica* crops are important and widespread species, and their seeds have medium size, and germinate rapidly and uniformly, and it is easy to break dormancy of them without scarification (Luo et al., 2019; Slankster et al., 2012; Rizwan et al., 2018). Therefore, they are used widely in seed germination test to study the phytotoxicity of substances (Kaur et al., 2017; Luo et al., 2018). The common species of *Brassica* crops include Chinese cabbage (*Brassica rapa* L.), edible rape (*Brassica napus* L.), and cabbage (*Brassica oleracea* L.) (Wang et al., 2011). Among the major-use antibiotics, such as fluoroquinolones, tetracyclines, and sulfonamides, more attention is paid to tetracyclines because they can disrupt the function of ribosomes in mitochondria and chloroplasts causing serious ecological risks (Carter et al., 2015; Moullan et al., 2015; Wang et al., 2015; Yu et al., 2017) and threatening human health through food chains (Christou et al., 2017; Liu et al., 2019; Walters et al., 2010; Wu et al., 2013). But so far there is a lack of research on tetracycline (TC), one of tetracyclines, to *Brassica* crop seeds, especially the comparison of different species and the analysis of species sensitivity difference. Therefore, their performance on evaluating the phytotoxicity of TC in seed germination test is worth studying.

The objectives of our study were to: (i) explore the potential endpoints in seed germination test with typical *Brassica* crops for evaluating the phytotoxicity of TC; (ii) establish the species sensitivity distribution (SSD) based on the toxic data on TC to *Brassica* crop seeds; and (iii) firstly analyze the source of sensitivity difference of seeds to TC from seed innate characteristics. Results from this study shed light on the development of seed germination test with sensitive species to investigate the phytotoxicity of TC to limit its level in the environment.

2. Materials and methods

2.1. Seeds and reagents

Nine cultivars of *Brassica* crop seeds were selected as model organisms, including Chinese cabbage (*Brassica rapa* L.), edible rape (*Brassica napus* L.), and cabbage (*Brassica oleracea* L.) with three cultivars for each species. These cultivars were ZS5 (*Brassica rapa* L. cv. Zaoshu No. 5), JQ3 (*Brassica rapa* L. cv. Jingqiu No. 3), and GLQZ (*Brassica rapa* L. cv. Gailiang Qingza) for Chinese cabbage; ZYZ19 (*Brassica napus* L. cv. Zhongyouza No. 19), ZY50 (*Brassica napus* L. cv. Zheyou No. 50), and ZY51 (*Brassica napus* L. cv. Zheyou No. 51) for edible rape; JF1 (*Brassica oleracea* L. cv. Jingfeng No. 1), XZG11 (*Brassica oleracea* L. cv. Xin Zhonggan No. 11), and ZG15 (*Brassica oleracea* L. cv. Zhonggan No. 15) for cabbage. All of the seeds were purchased from Chinese seed breeding companies. In order to use the tested seeds of the same cultivar with similar sizes, they were sieved to obtain those with the largest number between two consecutive standard sieves. Moreover, seeds were stored at 4 °C to break dormancy and prevent aging before use.

Tetracycline hydrochloride (CAS no.: 64-75-5; Molecular weight: 480.9) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide (NaOH) and ethanol were purchased from Sinopharm reagent Co., Ltd (Shanghai, China). Chemical reagents were of analytical grade, and deionized water was used in the test.

2.2. Treatments and control

The initial solution of TC at 1000 μM was prepared in the test. TC solutions at 1, 5, 10, 20, 50, 100 and 200 μM were obtained by diluting the initial solution with deionized water and pH was adjusted to 7.0 each by adding 0.01 M NaOH. The seeds exposed to TC solutions or deionized water (i.e. 0 μM TC) were set as the treatments or control (CK).

2.3. Seed germination test

Seed germination tests were performed according to the procedures in a previous study (Luo et al., 2019). Seeds were cleaned up with 75% ethanol (v/v) for 30 s and then washed with deionized water for three times. They were placed on a filter paper in a Petri dish (*Φ* 90 mm) added with 5 mL of the tested solution or deionized water and were put in an incubator at the suitable conditions of 25.0 ± 0.1 °C and darkness for the tests. At first a number of 160 seeds per dish were used for the treatments or control without replicates. After incubation for 24 h, twenty germinated seeds with similar sizes were selected and transferred to a new dish containing the same solution or water as before, and four replicates were carried out in the rest of total duration for incubation. The total duration for Chinese cabbage and edible rape were both 48 h and it for cabbage was 72 h. We gave some explanations about the operations and the durations for incubating seeds in the Discussion.

2.4. Measurement and analysis

2.4.1. Seed physical traits

Results of preliminary experiments indicated that 100-seed weight (m_{100}) of all the seeds in the study was ca. 0.2–0.4 mg. According to the results, the amounts of 0.09, 0.18, 0.36, 0.54 and 0.72 mg of seeds were weighted in duplicate and dispersed in *Φ* 90 mm Petri dishes to be photographed, respectively. The photographs were analyzed by ImageJ 1.50i software (US NIH) using its counting function to obtain the number of seeds. Finally, the m_{100} of seeds was calculated by the linear equation of the number versus the weight of seeds. Seed size (SS) was the thickness of a seed along its ridge and measured by a digital vernier caliper (Liu et al., 2012). The sizes of thirty individuals randomly selected from the amount of 0.36 g seeds (ca. one hundred seeds) were measured.

2.4.2. Seed storage reserves

Dried seeds were obtained by an oven-drying process at 100 °C for 3 h (Leubner-Metzger, 2005; Manz et al., 2005) and powdered for determining the content of total sugar (TS), total protein (TP), total lipid (TL) and ash with three replicates each. Briefly, TS was determined by dinitrosalicylic acid method using a commercial reagent kit purchased from Beijing Solarbio Science & Technology Co., Ltd., China. TP was determined by the Kjeldahl method according to China national standards: GB 5009.5–2016. TL was determined by the Soxhlet extraction method according to GB 5009.5–2016. Ash was determined by the burning-weighing method according to GB 5009.4–2016.

2.4.3. Seed germination indices

The initial germination time (IGT) was determined by successive observations and the number of germinated seeds was recorded after incubation for 24 h. At the end of test, seeds were photographed to measure the radicle length (RL) by using ImageJ 1.50i software (US NIH; Moubayidin et al., 2013). Then the germinated seeds were rinsed by deionized water and then dried by blotting papers. Finally, the cotyledon and embryonic axis were separated by a razor blade and weighed to obtain the fresh weight, respectively.

Operationally, a seed is defined as germination when its radicle is visible (Derek Bewley, 1997; Luo et al., 2018). RL was the distance from the bottom of hypocotyl to the tip of radicle (Hillis et al., 2011; Rusan et al., 2015). Besides IGT and RL, seed germination indices included the germination energy (GE), the radicle elongation rate (RER), the ratio of axis to cotyledon by fresh weight (RAC). Specifically, GE was the percentage of germinated seeds after a short-term incubation (Amooaghaie et al., 2015; Wei et al., 2014); and RER was the ratio of radicle length to elongation time.

2.5. Statistical analysis

Data ($n \geq 3$) were analyzed by one-way analysis of variance (ANOVA) where the means were compared by Tukey's honest significant difference (HSD) test using SPSS 22.0 software (IBM, USA). The *P*-value threshold for statistical significance was set at 0.05 (Benjamin et al., 2018). The values of the effective concentrations (EC_{20} and EC_{50}) and their 95% confidence intervals were obtained using TRAP 1.30a software (US EPAa) by a logistic model based on the dose-response relationship between TC and RL. Based on the values of EC_x, the data of species sensitivity distribution (SSD) curve were calculated by a log-probit model using SSD Generator V1 software (US EPAb; Guo et al., 2016a), as well as the hazardous concentration for 5% of species (HC_5) and for 50% of species (HC_{50}). Principal component analysis (PCA) was also performed by SPSS 22.0 software.

3. Results

3.1. Basic characteristics and germination indices of seeds

Results of this part were given in Table 1. In m_{100} and SS, there were big differences between Chinese cabbage and edible rape. Within the same species, the m_{100} of the cultivar ZS5 of Chinese cabbage was 0.2287 g, smaller than that of the other cultivars; and the m_{100} of ZYZ19 of edible rape was 0.6058 g, larger than that of the other ones. The SS of JQ3, ZYZ19 and ZG15 was 1.60, 2.13 and 1.67 mm, the largest one of Chinese cabbage, edible rape, and cabbage, respectively. We analyzed the content of TS, TP, TL and ash (representing minerals) in seed storage reserves. TL content was higher than TP content except for those in XZG11 and ZG15. TS content was lower than TP content, while it was higher than ash content. Among all the seeds, the content of SS and ash had no relationship with their species. However, the content of TP was decreased by the order of cabbage, Chinese cabbage, and edible rape, while the content of TL was increased by this order. Results of statistical analysis indicated that there were significant differences between all the content of seed storage reserves for any two cultivars of Chinese cabbage, only between the content of TL for cabbage and edible rape ($P < 0.05$). IGT was varied with seed species and cultivar. The lowest one was 8 h in ZS5 and JQ3, and the highest one was both 20 h in JF1 and XZG11. In seed germination after incubation for 24 h, the performance of Chinese cabbage was the best and its GE was not less than 90%. For edible rape and cabbage, the GE of their cultivars had big differences, which was from 57% to 97% and from 9% to 81%, respectively. In radicle elongation, RER also had big differences among species and among their cultivars, ranging from 0.18 to 1.09 mm h^{-1}. Furthermore, in the last part of results (Section 3.4) we analyzed the potential relationships between these indices.

3.2. Effects of TC on radicle elongation

Seed germination stage was unaffected by TC (data not shown), so only the effect of TC on seed post-germination stage (i.e. radicle elongation stage) was analyzed (Fig. 1). Obviously, RL decreased with the increase of TC concentration, indicating that TC had an inhibitory effect on radicle elongation. However, the RL of ZYZ19, ZY50 and XZG11 to TC at 1 μM were all higher than that of CK ($P < 0.05$) but without significant difference for ZY50 ($P > 0.05$). Compared with CK, RL decreased by 15.59%–78.95% for Chinese cabbage, 14.73%–69.13% for edible rape and 10.61%–75.15% for cabbage, respectively; and it was increased by 18.65%, 5.18% and 22.30% for ZYZ19, ZY50 and XZG11, respectively.

The quality of RL data, either among seed species or among cultivars, were different. After 48 h of incubation, the RL of ZS5, JQ3 and GLQZ from Chinese cabbage was reached at 8.52, 26.04 and 18.40 mm (Fig. 1a), respectively. While the RL of ZYZ19, ZY50 and ZY51 from edible rape was only reached at 9.00, 5.46 and 8.20 mm (Fig. 1b), respectively. For cabbage after 72 h of incubation, the RL of JF1, XZG11 and ZG15 was reached at 8.87, 10.21 and 15.46 mm (Fig. 1c), respectively. Thus, from the relative error of RL in measurement, it was the lowest for JQ3. In boxplot, the deviation from mean to median of data can reflect the deviation from its distribution to a normal distribution. In view of this point, the normality of RL data of JQ3 was the best. Moreover, from the length of the whisker on boxplot, the difference in RL data increased by the order of Chinese cabbage, edible rape, and cabbage. Overall, JQ3 was the best one of all the seeds in our study according to the quality of RL data.

Toxicological parameters based on the dose-response relationship between TC and RL were given in Table 2. Except for ZY50, JF1 and XZG11, the NOEC and LOEC of TC on the seeds were less than 1 μM and 1 μM, respectively. While the LOEC of XZG11 was the highest and was 20 μM, its EC_{20} and EC_{50} were the lowest and was 2.81 and 35.73 μM, respectively. Overall, the values of EC_{50} were 2.94–20.48 times the values of EC_{20}, indicating that EC_{20} reflected the sensitivity of seed to TC but EC_{50} reflected the extent of seed affected by TC. For example, in one cultivar of seeds, the lower value of EC_{20} is with the higher value of EC_{50}, such as ZY50. In two cultivars of seeds, the difference between the values of EC_{20} is much bigger than that of EC_{50}, such as ZS5 and JQ3, XZG11 and ZG15. Obviously, these phenomena can be attributed to the nonlinear dose-response relationship of TC and RL in the study (Fig. 1).

3.3. Effects of TC on endpoints about cotyledon and axis

There were no significant differences among the fresh weight of cotyledon of all seeds exposed to TC at different concentrations ($P > 0.05$) (Table S1). However, the fresh weight of cotyledon of JQ3 decreased with the increase of the concentration of TC, indicating that TC had an adverse effect on cotyledon growth but this effect was not significant. Axis was the rest of a germinated seed except for the

Table 1
Seed physical traits, storage reserves and germination indices.

Seed	m_{100} (g)	SS (mm) (n = 30)	TS (%)[a] (n = 3)	TP (%) (n = 3)	TL (%) (n = 3)	Ash (%) (n = 3)	IGT (h)	GE (%)	RER (mm h^{-1})
ZS5	0.2287	1.42 ± 0.12h[b]	7.39 ± 0.13bcd	31.83 ± 0.18d	36.68 ± 0.31e	5.29 ± 0.03bc	8	90	0.36
JQ3	0.3550	1.60 ± 0.10bg	6.47 ± 0.14efh	28.64 ± 0.01f	39.69 ± 0.18d	4.74 ± 0.00e	8	99	1.09
GLQZ	0.3395	1.42 ± 0.15hi	8.44 ± 0.08a	30.63 ± 0.72e	33.73 ± 0.31f	4.99 ± 0.07d	12	94	0.77
ZYZ19	0.6058	2.13 ± 0.11a	7.43 ± 0.30bc	24.69 ± 0.12g	42.54 ± 0.17c	3.85 ± 0.06hi	18	63	0.37
ZY50	0.3866	1.68 ± 0.14b	7.69 ± 0.08b	21.17 ± 0.04h	46.02 ± 0.26a	3.99 ± 0.07gh	18	57	0.23
ZY51	0.3888	1.62 ± 0.18bf	7.11 ± 0.38bcde	21.91 ± 0.19hi	44.27 ± 0.08b	4.02 ± 0.05g	14	97	0.34
JF1	0.3803	1.66 ± 0.18bd	6.84 ± 0.15cdef	32.74 ± 0.01bc	33.39 ± 0.18 fg	4.64 ± 0.08ef	20	19	0.18
XZG11	0.4041	1.64 ± 0.17be	6.77 ± 0.14defg	33.43 ± 0.50b	30.37 ± 0.29i	5.58 ± 0.07a	20	9	0.21
ZG15	0.3768	1.67 ± 0.14bc	6.43 ± 0.35fi	35.68 ± 0.25a	32.33 ± 0.22h	5.43 ± 0.03 ab	14	81	0.32

[a] By dry weight.

[b] Data were expressed as mean ± SD. At first the mean values from large to small were marked with lowercase letters in ascending order and then the results of significance analysis were marked. The data labelled with a same lowercase letter indicated that there were no significant differences among them at $P < 0.05$.

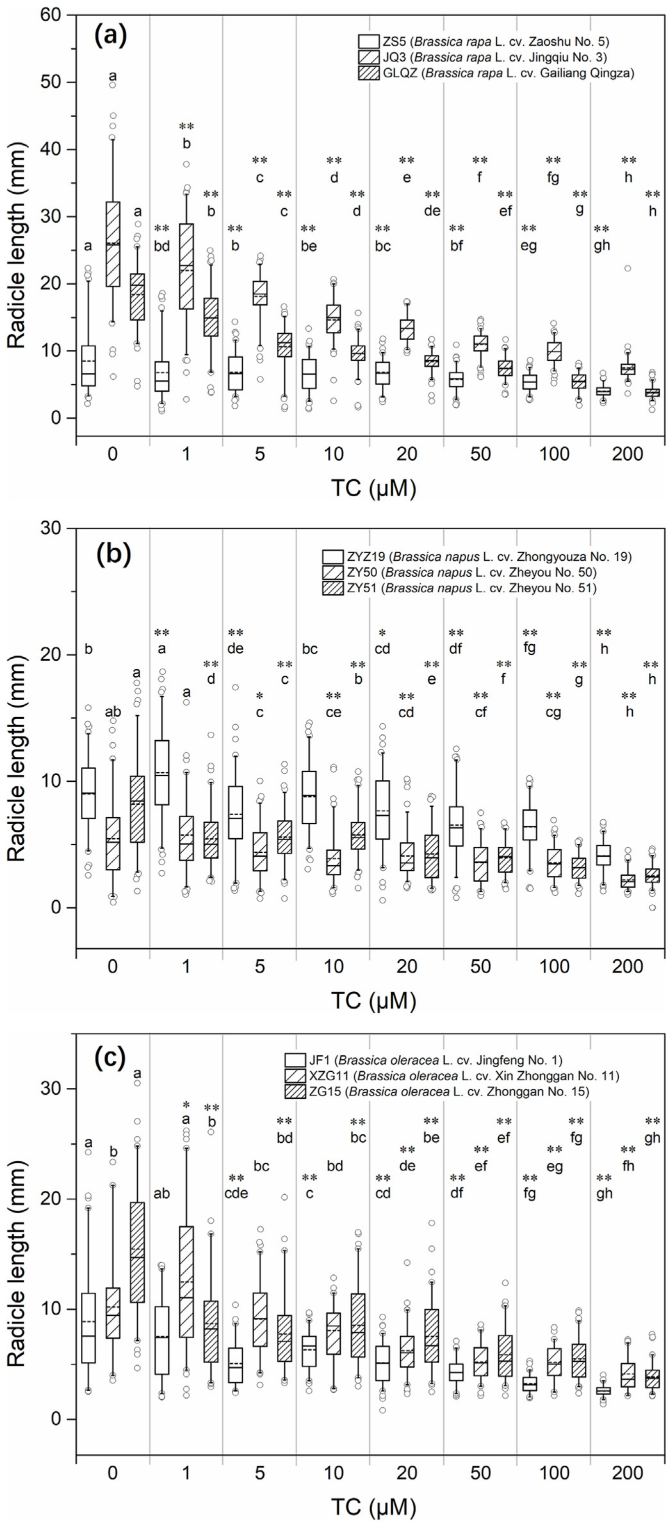

Fig. 1. Radicle length (RL) of (a) Chinese cabbage, (b) edible rape, and (c) cabbage seeds exposed to different concentrations of tetracycline (TC). The solid and dotted lines in the boxes, the lower and upper edges of the boxes, the lower and upper ends of the whiskers on the boxes, the circles outside the boxes represented the values of median and mean, 25th and 75th percentiles, 5th and 95th percentiles, and outliers more than 1.5 times the box height of RL data (n = 80), respectively. At first the means values from most to least were marked with lowercase letters in ascending order, and then the results of significance analysis were marked. The data were labelled with a same lowercase letter, indicating that there were no significant differences among them at $P < 0.05$; single and two asterisks indicate that they have significant differences compared with the control (CK) at $P < 0.05$ and at $P < 0.01$, respectively.

Table 2
Toxicological parameters based on the dose-response relationship between tetracycline (TC) and radicle length (RL).

Seed	NOEC (μM)	LOEC (μM)	EC_{20} (μM)	EC_{50} (μM)
ZS5	< 1	1	91.63 (72.05–116.54)[a]	269.77 (198.92–365.85)
JQ3	< 1	1	4.98 (2.11–11.75)	55.34 (34.08–89.87)
GLQZ	< 1	1	3.51 (1.40–8.78)	36.07 (22.36–58.19)
ZYZ19	< 1	1	39.63 (11.40–137.83)	194.93 (60.93–623.65)
ZY50	1	5	6.10 (1.37–27.15)	124.94 (41.96–372.03)
ZY51	< 1	1	30.77 (14.87–63.65)	143.49 (82.45–249.69)
JF1	1	5	25.72 (7.41–89.21)	117.56 (49.58–278.74)
XZG11	10	20	2.81 (0.77–10.18)	35.73 (18.66–68.41)
ZG15	< 1	1	42.85 (24.61–74.61)	169.31 (107.78–265.95)

[a] Values in parentheses were 95% confidence intervals.

cotyledon and consisted of hypocotyl and radicle in our study. The effect of TC on axis was bigger than that of TC on cotyledon. Except for ZYZ19 and XZG11, the LOEC based on the endpoint of the fresh weight of axis was observed in all seeds. The lowest and highest values of LOEC were both from cabbage, which indicated that the sensitivity difference of this seed to TC was relatively large. Compared with axis, the values of LOEC based on RAC were lower except for those of JQ3, GLQZ, XZG11 and ZG15 with the same values. Thus, RAC was more sensitive than axis to be affected by TC. For a limited number of seeds, the test result inevitably varied with the weight of seeds which mainly depended on the weight of their cotyledons. However, RAC reduced the influence of the weight difference of cotyledon on the growth of axis, which was also verified in our study. Therefore, RAC can be chosen as a toxic endpoint besides RL in seed germination test.

3.4. SSD based on endpoint of RL

In this study the tested seeds were all from the genus of *Brassica*. In order to study the inter- and intra-specific differences of the seeds to TC, three species of this genus and three cultivars of each species were selected, respectively. Judging from the values of EC_{20}, the sensitivity of seeds to TC decreased by the order of XZG11, GLQZ, JQ3, ZY50, JF1, ZY51, ZYZ19, ZG15 and ZS5 (Fig. S1); and from EC_{50}, the sensitivity decreased by the order of XZG11, GLQZ, JQ3, JF1, ZY50, ZY51, ZG15, ZYZ19 and ZS5 (Fig. 2). It was obvious that, whatever it was based on, the order of the sensitivity of the seeds within the same species to TC was unchanged but not that among different species. Overall, the

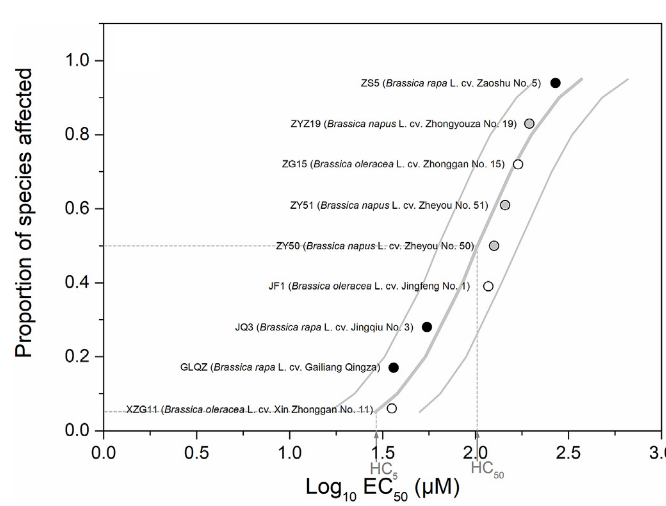

Fig. 2. Species sensitivity distribution (SSD) curve based on EC_{50} of tetracycline (TC) to *Brassica* crop seeds. Fine lines represented its 95% confidence intervals.

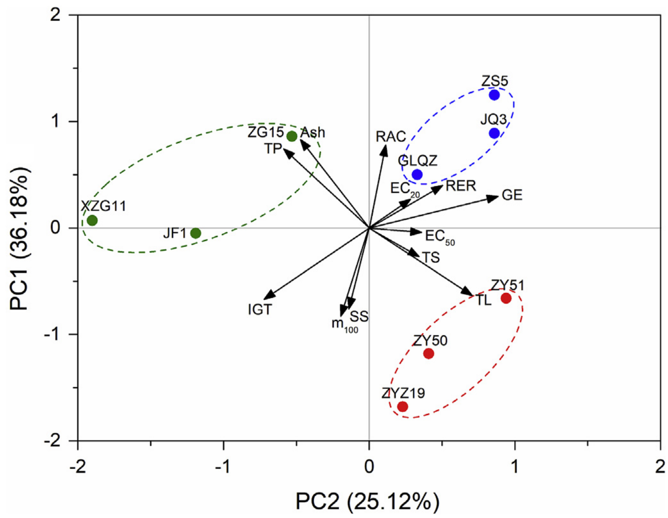

Fig. 3. Source analysis of the sensitivity difference of *Brassica* crop seeds to tetracycline (TC) by principal component analysis (PCA).

species-dependent sensitivity of seeds to TC was decreased by the order of Chinese cabbage, cabbage, and edible rape, while the difference in the sensitivity of the cultivars from the same species increased by the order of edible rape, Chinese cabbage, and cabbage. Moreover, inferred from the SSD curve plotted by EC_{50}, the HC_5 and HC_{50} of TC to all species were 28.55 and 103.29 μM, respectively; and by EC_{20}, they were 1.61 and 14.93 μM, respectively. However, the lowest concentration of the HC_5 and HC_{50} of TC was far more than the concentrations of TC at environmentally relevant concentrations (ng L^{-1} − μg L^{-1}), indicating that TC alone had no environmental risk at low concentrations.

3.5. Source analysis of the sensitivity difference of seeds to TC

In this part we analyzed the potential relationships between these indices about seed physical traits, storage reserves, germination indices and toxic endpoints by PCA to elucidate the source of the sensitivity difference of seeds to TC (Fig. 3). Results showed that the first two principal components (PCs) accounted for 61.30% of total variance and were considered enough for further analysis. At the first principal component (PC1), ash, RAC, TP and RER had high positive loads, and m_{100}, SS, IGT and TL had high negative loads. While at the second principal component (PC2), GE, TL, RER, EC_{50} and TS had high positive loads, and IGT, TP and ash had high negative loads. From the score plots of seeds, clear clustering and separating of each species were observed according to their local areas, which indicated that there were distinct differences among these species. In addition, the seeds of Chinese cabbage had positive relationships with PC1 and PC2, and the seeds of edible rape had negative relationships with PC1 but had positive relationships with PC2. The result of cabbage were contrary to edible rape, indicating that there were big differences among the two species. Moreover, seen from the sizes of their local areas, the intra-species difference of seeds was decreased by the order of cabbage, edible rape, and Chinese cabbage. Overall, although EC_x had small loads at both of PC1 and PC2, EC_{20} was relevant to the indices of seed physical traits (m_{100} and SS) and the germination indices (RER and IGT) while EC_{50} was relevant to the indices of seed storage reserves (TS, TL, TP and ash). Therefore, the sensitivity of seed to TC is dependent on the performances of seed physical traits and germination, while the extent of seed affected by TC is dependent on the composition of seed storage reserves.

4. Discussion

Seeds of *Brassica* crops belong to endospermless dicotyledons which are composed of testa (i.e. seed coat) and embryo (Ren and Bewley, 1998; Weitbrecht et al., 2011). From the structure of testa, it consists of epidermal layer, palisade layer, and aleurone layer (Ran et al., 2017). Besides the short duration of seed germination, the protective role of testa was the main reason to explain that the seeds at germination stage before testa rupture was not sensitive to TC at the tested concentrations (ca. 0.5 mg L^{-1}–100 mg L^{-1}) in our study. The result supported the fact that radicle elongation is much more sensitive than seed germination to antibiotics (Luo et al., 2019; Pan and Chu, 2016). Embryo includes cotyledon and axis, while axis includes hypocotyl and radicle. Seed germination happens at the stage of plant skotomorphogenesis in the dark when radicle is the rapidly growing organ and sensitive to TC. For edible rape (ZYZ19 and ZY50), as well as cabbage (XZG11), TC induced hormesis in the radicle elongation of these seeds at 1 μM. As our study showed, the hormesis of antibiotics on crops existed widely in different species, which could be attributed to multiple adaptive mechanisms of plants to low levels of stresses (Agathokleous et al., 2018; Poschenrieder et al., 2013). However, at the later stage of seedling establishment where plants enter the stage of photomorphogenesis in the light, hypocotyl and cotyledon are the rapidly growing organs (Yang and Benning, 2018). Therefore, the test to investigate the effect of TC on seed germination stage of plants should be terminated before plants develop into the stage of photo-autotrophic growth to eliminate the confounding factors from the latter stage. Considering this respect, the test duration of each cultivar of the seeds in our study was dependent on their specific germination characteristics.

Learning from the root:shoot ratio (R:S ratio), the ratio of above-ground to belowground biomass to evaluate plant health during the period of photo-autotrophic growth (Gong et al., 2017; Minden et al., 2017), we firstly adopted the RAC that is the ratio of axis to cotyledon by fresh weight at radicle elongation stage. RAC not only evaluates the growth of axis relative to cotyledon but also reduces the disturbance from seed cotyledons with different weights on the growth of axis. Moreover, our study indicated that RAC was better than the separated cotyledon and axis to reflect the phytotoxicity of TC. Therefore, it is an alternative endpoint when RL can not be measured with enough accuracy, such as in the case where there is not an obvious boundary between hypocotyl and radicle.

To date, SSD method is applied to evaluate potential risks of antibiotics in waters (Guo et al., 2016a, 2016b; Zhang et al., 2014) but not in soils as other pollutants (Ding et al., 2016; Kwak et al., 2017). In general, the prerequisite for using SSD method is that test organisms should be not less than five species from at least three different taxonomic groups (Kwak et al., 2018). Thus, enough sample size and representativeness of this method can be guaranteed (Newman et al., 2000). Only one taxonomic group, i.e. plant, was selected for establishing SSD in our study, so the HC_5 and HC_{50} inferred from SSD lacked the representativeness. However, in another sense, our study showed that there was big difference in the sensitivity of the seeds from inter- and intra-species to TC, indicating that it is necessary to select sensitive species to establish SSD to infer HC_x.

From the aspect of substance metabolism, seed germination is the biochemical processes where storage reserves of a seed are mobilized and utilized for its germination (Derek Bewley, 1997; Graham, 2008). This belongs to the stage of plant heterotrophic growth (Avelino et al., 2017; Gommers and Monte, 2018). Seed storage reserves are mainly in cotyledons where the starches, proteins, lipids, and minerals are stored in starch grains, protein bodies, lipid bodies, and electron dense crystals, respectively (Kuang et al., 2000; Ran et al., 2017). However, starch grains are generally disappeared in the mature seeds of *Brassica* crops (Kuang et al., 2000; Qouta et al., 1991), so we did not measure the content of starch. The composition of seed storage reserves showed that the seeds of Chinese cabbage, and cabbage were rich in lipids as edible rape, and proteins were the second largest storage reserves in these seeds. Qouta et al. (1991) found that protein content decreased rapidly during the first two days after imbibition. After day two, lipid content began to decrease and was exhausted by day five. However, the content of soluble sugar remained unchanged during this period. Thus, we inferred that proteins and lipids, the first two largest seed storage reserves, played important roles in seed germination process. Seed germination is also related to its physical traits besides the composition of storage reserves. Previous findings revealed that small seeds were more inclined to germinate than big seeds for spring wheat (*Triticum aestivum* L.) (Lafond and Baker, 1986), and the faster the seeds germinated, the higher the content of soluble sugar and lipid they had for sunflower (*Helianthus annuus* L.) (Munshi et al., 2007). Likewise, our study also supported that there were certain relationships between seed germination and multiple characteristics. For the seeds from *Brassica* crops in our study, there were positive or negative correlations between seed germination indices (such as IGT, GE and RER) and physical traits (such as m_{100} and SS). Although the content of seed storage reserves had relatively weak relationships with other indices, they had relatively strong relationships with each other. By analyzing the source of the sensitivity difference of seeds to TC, EC_{20} was relevant to seed physical traits and germination indices, and EC_{50} was relevant to the composition of seed storage reserves. Our study provided some available information on a novel idea that the composition of storage reserves could influence the sensitivity or resistance of seeds to pollutants (Kaur et al., 2017). But the sample size of our study was limited. If the number of species from the same genus is increased, or some species of seeds from different families are adopted, results may be obtained with a good regularity and a high level of representativeness.

5. Conclusions and perspectives

In all of the endpoints derived from morphological changes of seeds at germination and post-germination stages, RL was the most sensitive indicator to TC. Besides RL, RAC was better than separated axis and cotyledon to reflect the phytotoxicity of TC. Judging from EC_x based on RL, the species-dependent sensitivity of seeds to TC was decreased by the order of Chinese cabbage, cabbage, and edible rape, while the difference in the sensitivity of the cultivars from the same species increased by the order of edible rape, Chinese cabbage, and cabbage. Overall, Chinese cabbage was the most sensitive species in the study. HC_x derived from SSD curve indicated that TC alone had no environmental risk at low μg L^{-1} levels. EC_{20} can reflect the sensitivity of a seed to TC and it is relevant to seed physical traits and germination indices. While EC_{50} can reflect the extent of TC on a seed and it is relevant to the composition of seed storage reserves. Therefore, it is a rapid and feasible approach to select sensitive seeds to TC from different families of typical crops according to seed innate characteristics, especially the composition of storage reserves, at large scale, but need further studies.

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Appendix A. Supplementary data

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