Effects of Oxytetracycline on Growth and Chlorophyll Fluorescence in Rape (*Brassica campestris* L.)

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Abstract

This study aims to investigate the effects of oxytetracycline on growth and photosynthetic capacity of rape (*Brassica campestris* L.). Four levels of oxytetracycline (0, 10, 100, and 200 mg kg^{-1}) were added to the potted soil. Chlorophyll fluorescence parameters induced by five levels (90, 190, 420, 820, and 1,500 µmol photons m^{-2} s^{-1}) of PAR (PAR, photosynthetically active radiation) were measured. Plant growth indices and leaf traits were also determined. Electron transport rate increased along with the increase of PAR, but an opposite trend was found for the effective quantum yield of PSII. The quantum yield of light-induced non-photochemical fluorescence quenching in higher oxytetracycline treatments (100 and 200 mg kg^{-1}) gradually increased when PAR increased from 90 to 820 µmol photons m^{-2} s^{-1}, but then declined under higher PAR gradients. The fractions of quantum yield of non-light-induced non-photochemical fluorescence quenching in PSII were significantly higher in all the oxytetracycline treatments than in the control. Oxytetracycline exposure was also found to alter the energy distribution in the photosynthetic electron transport chain. This study showed that oxytetracycline exposure evidently diminished the photosynthetic capacity of rape, which was further supported by the observations that growth indices and leaf traits were significantly inhibited by oxytetracycline.

Keywords: oxytetracycline, chlorophyll fluorescence, rape, soil pollution, growth

Introduction

Antibiotics, widely used by people or livestock to cure disease, are often excreted directly in original chemical form through urine and feces [1]. Antibiotics can persist in the environment owing to their stable physical and chemical properties. The accumulation rate of residual antibiotics has gradually exceeded the degradation rate in soil [2], which may cause indisputable environmental pollution [3]. For decades, various residual antibiotics were detected in different environmental and plant samples, including soil, surface water, underground

drainage, groundwater, air, food crops (such as wheat), and vegetables [4-6]. It has been a growing issue that plants, animals, and microbes are facing risk of exposure to antibiotics with low doses for long-term and even the whole life history in an ecosystem [7-8]. Antibiotics are an emerging kind of pollutant in soil, which recently has received growing concern in the research field of ecotoxicology.

Tetracyclines are a class of broad-spectrum antibiotics used by humans and animals with the highest consumption in the world. The accumulation of tetracyclines in farmland through the application of manure is increasing annually. In Europe, the value of tetracycline concentration in samples of pig manure was < 46 mg kg^{-1} [9]. Average contents of tetracycline within 0-10 cm soil layer from Germany farmland and meadow were 4 mg kg^{-1} and 0.86 mg kg^{-1}, respectively [10]. The detection rate of oxytetracycline was the highest of the tetracycline antibiotics in China [11]. The range of tetracycline concentration in pig manure samples was 1-100 mg kg^{-1} in China [12]. Similarly, residual tetracycline content in farmland soil was detected as 2.669 mg kg^{-1} in the eastern region of China [13]. The highest concentration of residual oxytetracycline in 0-40 cm soil due to fertilization with animal excreta was 2.683 mg kg^{-1} in northern China, and the maximum concentration reached 183.50 mg kg^{-1} in animal manure [14].

Residual oxytetracycline in soil has effects of acute or chronic toxicity on organisms [15]. It is well known that tetracyclines in soil can affect plant growth and photosynthesis capacity of *Phaseolus vulgaris* [16], *Oryza sativa* [17], *Triticum aestivum* [18], and *Iberis sempervirens* [19]. Certain ecological toxicity of the tetracyclines on plant roots, stems, and leaves were also confirmed with some hydroponic research [20]. Recently, research has focused on understanding and defining the ecological toxicity of tetracyclines on crops.

Brassica campestris "Zheshuang 72" is an excellent cultivar of rape widely planted in eastern China. The growth of such a crop being threatened by oxytetracycline is increasing. So far, studies of rape have focused mainly on crop cultivation [21], bioenergetics [22], genetics and breeding science [23-24], and genomics [25-26]. Recently, various pollutants such as ozone and heavy metals and their effect on rape have been investigated in many reports [27-30]. However, little is known about the ecological toxicity of soil oxytetracycline on rape [31].

Photosynthesis is an important biochemical process that converts light energy into chemical energy. Chlorophyll fluorescence produced by the PSII reaction center in plant leaves is closely related to the process of photosynthesis reaction. In general, the fraction of energy dissipation through producing chlorophyll fluorescence is very slight [32]. It is well known that there are mainly three allocation paths of light energy captured by the PSII reaction center: (1) to drive the electrons from the reaction center P_{680} to Q_A represented by effective quantum yield of photosystem II (Φ_{PSII}); (2) light-induced non-photochemical quenching (Φ_{NPQ}); and (3) non-light-induced non-photochemical quenching (Φ_{NO}) [33-35]. There is a dynamic equilibrium under different photosynthetically active radiation (PAR) conditions, and the balance equation is shown as $\Phi_{PSII} + \Phi_{NPQ} + \Phi_{NO} = 1$. Chlorophyll fluorescence is a rapid, and non-traumatic probe to study the photosynthetic capacity of plants [36-37]. It is also a useful technique for revealing the photosynthetic physiological law of plants in adversity. At present, chlorophyll fluorescence induction kinetics curve analysis has been extensively used to assess the photosynthetic system status of plants growing under adverse environments from the perspective of energy allocation and balance [38]. Some studies have revealed that antibiotics can have ecotoxicological impacts on the photosynthesis of photosynthetic organisms [39-40].

Overall, it is urgent that we explore the potential ecological toxicity of oxytetracycline on Zheshuang 72 as it relates to photosynthesis. In this study, we attempt 1) to unseal the growth characteristics of rape affected by oxytetracycline content in soil, 2) to disclose the features of energy distribution in the photosynthetic electron transport chain in leaves of rape under oxytetracycline exposure, and 3) to evaluate the ecotoxicity of the oxytetracycline on rape with the characteristics of photosynthetic activity and photo-protective capacity. The results will be helpful for understanding the ecotoxicology of environmental antibiotics on profitable crops.

Material and Methods

Exposure Assays

Oxytetracycline hydrochloride (≥98.9% purity, standard grade, Merck & Co., Inc., Germany) was purchased from Hefei Meifeng Chemical Ltd. Co. (Hefei, China). Zheshuang 72 was purchased in a local seed market in Hefei. First of all, we cultured the seedlings of rape to draw out the first true leaf for potting. The air-dried soil samples were sieved through a 4-mm mesh sieve and placed into plastic pots with an upper diameter of 22.0 cm, a lower diameter of 18.0 cm, and a depth of 20.0 cm, and each pot was filled with about 3.5 kg soil mixed uniformly with the specified content of the oxytetracycline. Four level contents of the oxytetracycline were 0, 10, 100, and 200 mg kg^{-1} marked with CK, A, B, and C, respectively. The bulk density of soil is 0.87 g cm^{-3}, and available N, P, K, and organic matter content were 39.22 mg kg^{-1}, 16.41 mg kg^{-1}, 41.73 mg kg^{-1}, and 22.28 g kg^{-1}, respectively. Three seedlings of rape with similar size were randomly allocated into each pot. Each treatment was replicated three times in an artificial climate chamber at Hefei Normal University (13 h light, 25ºC, per day) with PAR of 200 μmol photons m^{-2} s^{-1} and relative humidity of 55-65%. The plants were watered every day to keep the soil moist. Cultivation experiments were conducted from 1 December 2015 to 1 March 2016, and the following parameters/indices were determined at the end of the cultivation phase.

Plant Growth Parameter Measurements

After non-traumatic leaf traits determination and Fluorescence induction kinetics curve analysis, total biomass for each plant, including roots, stems, and leaves, was weighed with an electronic balance after drying at 80ºC for 48 h ($n = 9$ for each treatment), and the number of rape nodes of each plant under different treatments was counted ($n = 9$ for each treatment).

Leaf Traits Determination

In order to ensure the comparability of the data, we analyzed the third fully expanded leaf from the upper end of each plant under each of four treatments to be the measurement unit in this paper ($n = 9$). The leaves were traced for leaf traits determination.

First, after fully dark adapted for 10 h, potential photosynthetical capacity represented as maximum quantum yield (Fv/Fm) of PSII in leaf was measured with a portable Junior-PAM (Walz, Germany), which applies a modulated blue light (LED-lamp peaking at 450 nm) as a source for measuring actinic and saturating light. Each target leaf was fixed in a magnetic leaf clip and kept at a distance of 1 mm from leaf surface to the tip of the 1.5 mm-diameter plastic fiber optics. The value of F_v/F_m was calculated from the equation:

$$F_v/F_m = (F_m - F_o)/F_m$$

…where F_o, F_m, and F_v are initial, maximum, and variable chlorophyll fluorescence, respectively. F_o was assessed with a weak modulated pulse (5 µmol photons m^{-2} s^{-1}), and F_m, corresponding to all PSII centers in the closed state, was induced by a 0.7 s pulse of saturating modulated blue light (10,000 µmol photons m^{-2} s^{-1}).

Next, relative chlorophyll content of leaf (represented as SPAD) was determined using a SPAD-502 Plus chlorophyll meter (Minolta Japan, Konica). SPAD readings provide a noninvasive surrogate method for surveying leaf chlorophyll concentration [41]. The average of three SPAD readings for each leaf was recorded. Finally, leaf length and leaf width were determined with a digital vernier caliper.

Fluorescence Induction Kinetics Curve Analysis

The above leaves were also traced for chlorophyll fluorescence measurements ($n = 9$). Chlorophyll fluorescence induction kinetics curve analysis was also performed with the portable Junior-PAM. For each leaf, electron transport rate (ETR), effective quantum yield of photosystem II (Φ_{PSII}), quantum yield of light-induced non-photochemical fluorescence quenching (Φ_{NPQ}), and quantum yield of non-light induced non-photochemical fluorescence quenching (Φ_{NO}) were determined under five levels of actinic light (PAR = 90, 190, 420, 820, and 1,500 µmol photons m^{-2} s^{-1}) with an initial measuring procedure of instrument from 08:30 to 11:30. The value of Φ_{PSII} at each PAR gradient was calculated from the equation:

$$\Phi_{PSII} = (F_m' - F)/F_m'$$

…where F is the fluorescence yield in real time and F_m' is the maximum fluorescence yield of a certain actinic light-adapted leaf. ETR was calculated as:

$$\Phi_{PSII} \times PAR \times a \times b$$

…where $a = 0.84$ is the leaf absorbance and $b = 0.5$ reflects the fraction of photons absorbed by PSII [35]. The values of Φ_{NPQ} and Φ_{NO} were calculated according to Kramer et al. [34].

In addition, the values of Fv/Fm, ETR, Φ_{PSII}, and Φ_{NPQ} were calculated automatically by the instrument.

Statistical Analyses

Data were presented as mean ± SE. Differences of growth parameters, relative chlorophyll content, and chlorophyll fluorescence parameters among treatments were assessed with a one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test in IBM SPSS statistics 23.0 software package (IBM, Chicago, USA). Significant differences were marked among treatments at the 0.05 level. Relationships between oxytetracycline content and growth parameters, leaf traits, and chlorophyll fluorescence parameters with a linear regression model. Data were log or arc-sine transformed before analysis to meet assumptions of normality and homogeneity of variance. Figures were drawn with Origin 8.5 (Origin Lab, USA).

Results and Discussion

Oxytetracycline Effects on Plant Growth

The total biomass dry weights of each rape plant showed a decreasing trend along with the increase of oxytetracycline concentrations (Fig. 1a), but oxytetracycline treatment at 10 mg kg^{-1} and 100 mg kg^{-1} showed
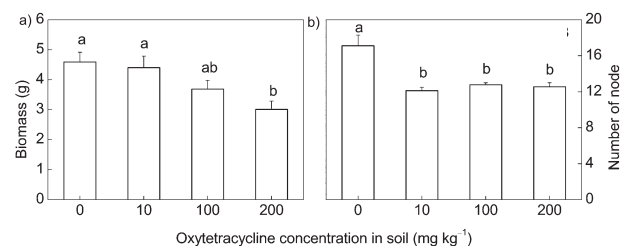

Fig. 1 Effects of oxytetracycline on biomass a) and nodes b) of rape (mean ± SE, $n = 9$). Different letters above bars indicate significant differences ($p<0.05$)
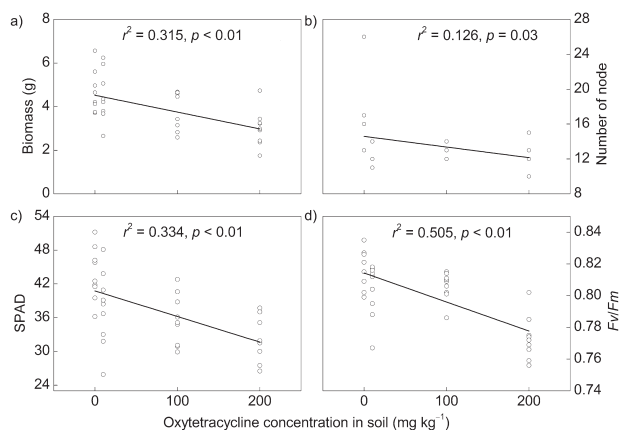
Fig. 2 Correlations fitting with linear regression between oxytetracycline concentrations and biomass a), and node number b), and relative chlorophyll content (SPAD) c), and maximum quantum yield of PSII (Fv/Fm) d) ($n = 36$).

no obvious effects on the biomass ($p>0.05$ each). However, the plants cultured with 200 mg kg^{-1} oxytetracycline had significantly lower total biomass dry weight than that of control ($p<0.05$), indicating that oxytetracycline exposure at high concentrations may have an inhibition effect on rape growth. Each treatment had fewer nodes than the control, but no significant differences were observed among the three treatments (Fig. 1b). Furthermore, both the biomass ($r^2 = 0.315$, $p<0.01$) and node number ($r^2 = 0.126$, $p = 0.03$) showed negative correlations with the soil oxytetracycline contents, indicating that the oxytetracycline in soil can inhibit the biomass accumulation and node number increase (Figs 2a-b). These results are supported by previous studies, since Batchelder [16] reported that oxytetracycline could decrease the plant biomass and heights of *Phaseolus vulgaris*. Liu et al. [17] also reported that tetracycline and chlortetracycline have an inhibitory effect on the growth of *Oryza sativa* (rice). The slight inhibitory effect of tetracyclines in soil on higher plants was also unsealed in previous studies [19, 31], which might be attributed to their strong adsorption onto soil components [42]. The results of the present study were in accordance with these reports.

Changes in Leaf Traits after
Oxytetracycline Treatment

In this study, rape exposed to oxytetracycline at 200 mg kg^{-1} had more leaves of yellow discoloration, aging, and shedding than the control, which agrees with a number of previous studies [19-20, 43-44]. SPAD in leaves of rape were declined by 14.1%, 18.6%, and 26.5% at 10 mg kg^{-1}, 100 mg kg^{-1}, and 200 mg kg^{-1} oxytetracycline treatments compared to that of the control, respectively (Fig. 3a); $p<0.05$ each. The maximum quantum yields (*Fv/Fm*) in rape leaves were also slightly decreased by 1.8%, 1.4%, and 5.5% in three sequential treatment groups (Fig. 3b). Oxytetracycline exposure at different

concentrations showed no effects on leaf size (including leaf length and leaf width) ($p>0.05$; Figs 3c-d).

The oxytetracycline absorbed by the plant from the root can inhibit chloroplast translation activity and chloroplast synthase activity because its chemical structure is similar to chloroplast photosynthetic enzyme [19-20], which may account for the inhibition of the photosynthetic capacity of plants. In the present study, *Fv/Fm*, indicating the maximum quantum yield of photosystem II, was used to assess the potential photosynthetic capacity of the plant. *Fv/Fm* in rape leaf exposed to 200 mg kg^{-1} oxytetracycline was significantly lower than that of the control. Marco et al. [19] found that the treatment of *Iberis sempervirens* with 100 or 200 mg L^{-1} tetracycline for 30 d induced the decrease of chloroplast photosynthetic efficiency since photosynthetic pigment contents, including chlorophyll *a*, *b*, and carotenoid content, decreased significantly in the treated plants, which was also evidenced by findings of chlorotic phenotype of some treated plant leaves. A similar result has also been documented regarding the cycloheximide effect on photosynthesis of *Phyllostachys edulis* [45]. Other antibiotics, such as fosmidomycin, have also been found to reduce the net photosynthesis rates of both white poplar (*Populus alba*) and tobacco (*Nicotiana tabacum*) [46]. Oxytetracycline exposure at 0-200 mg kg^{-1} posed no obvious effects on the morphology of the leaves, but led to an irreversible moderate decrease in potential photosynthetic capacity of rape.

Soil oxytetracycline contents showed strongly negative correlations with *Fv/Fm* ($r^2 = 0.505$, $p<0.01$), while weakly negative correlations with SPAD ($r^2 = 0.334$, $p<0.01$; Figs 2c-d), revealing that the *Fv/Fm* and SPAD in rape leaves could be inhibited by soil oxytetracycline (Fig. 2). The response of *Fv/Fm* to adversity stress is often shown to be a very slow descending process [47].
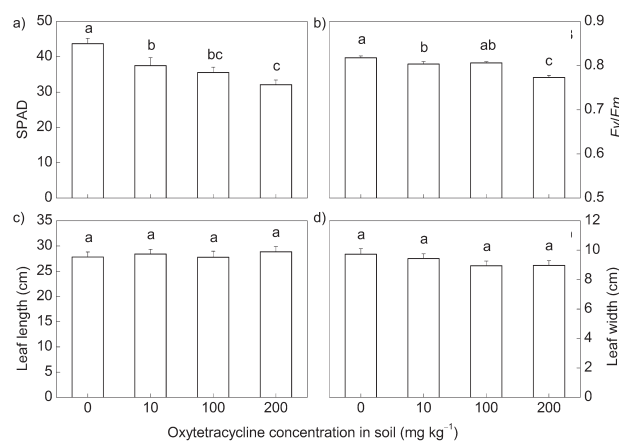

Fig. 3. Effects of oxytetracycline on relative chlorophyll content (SPAD) a), maximum quantum yield of PSII (*Fv/Fm*) b), and leaf size (c and d) of rape (mean ± SE, $n = 9$). Different letters above bars indicate significant differences.

Changes of Chlorophyll Fluorescence Parameters after Oxytetracycline Treatment

After the oxytetracycline treatment, the rape leaves had higher *ETR* of PSII for each treatment at higher PAR ($p<0.05$ each; Fig. 4a). Under the PAR gradients over 90 µmol photons m^{-2} s^{-1}, *ETR* was declined significantly due to oxytetracycline exposure in soil. The maximum *ETR* among the four treatments was obtained at the highest PAR (1,500 µmol photons m^{-2} s^{-1}).

Peak value of *ETR* will be occurring when the photosynthesis of plants reaches the saturation status. *ETR* will then decline, which may be accompanied by the phenomenon of light inhibition and even light damage with the PAR increase [48]. Possell et al. [46] found that the *ETR* in leaves of white poplar (*P. alba*) was significantly lower in fosmidomycin exposure treatments than in the control with PAR at 1,000 µmol photons m^{-2} s^{-1}. There were apparent non-stomatal inhibitions of photosynthesis, as demonstrated by the reductions of *ETR* in white poplar (*P. alba*) [46], which is similar to our findings in this study. The inhibition of *ETR* of PSII in rape leaves indicated that oxytetracycline exposure posed evident effects on photosynthesis capacity.

Φ_{PSII} was reduced significantly with the PAR increase in each treatment (Fig. 4b). For the control and 10 mg kg^{-1} oxytetracycline treatment, Φ_{NPQ} of rape leaves increased gradually during the increasing periods of PAR from 0-1,500 µmol photons m^{-2} s^{-1}. For the other two treatments, with the PAR increase Φ_{NPQ} increased gradually, and reached the maximum values at 820 µmol photons m^{-2} s^{-1}, but then sharply declined at 1,500 µmol photons m^{-2} s^{-1} (Fig. 4c). A continuous increasing trend was observed for Φ_{NO} of rape leaves in response to the PAR increase at each oxytetracycline exposure level (Fig. 4d).

Φ_{PSII} means actual primary light energy captures efficiency of PSII reaction center, and can be used to quickly determine operating efficiency of plant PSII reaction center [36]. Φ_{PSII} showed a decreasing trend with the increase of soil oxytetracycline concentration under each PAR gradient, indicating that the operating efficiency of PSII reaction center in rape leaves was significantly inhibited by oxytetracycline exposure. This might be attributed to a reduction of PSII electron output caused by oxytetracycline exposure. When PAR was up to 190 µmol photons m^{-2} s^{-1}, the decline of Φ_{PSII} caused by oxytetracycline exposure was significant. With PAR over 420 µmol photons m^{-2} s^{-1}, Φ_{PSII} in rape leaves showed more sensitivity to oxytetracycline exposure than under the other PAR gradients according to the *F* values in ANOVA (data not shown).

Φ_{NPQ} is an important index of plant photo-protective capacity. Higher Φ_{NPQ} indicates that light energy captured by plants is excessive, and redundancy excitation energy can be eliminated with a regulatory heat dissipation mechanism to avoid potential damage for the photosynthetic unit [34]. Interestingly, when PAR was below 820 µmol photons m^{-2} s^{-1}, Φ_{NPQ} of rape leaves were significantly higher in all three treatments than in the control treatment ($p<0.05$ each), revealing that light energy captured by rape under oxytetracycline exposure was excessive, but the redundancy excitation energy still could be eliminated through heat dissipation. By contrast, when PAR was up to 820 µmol photons m^{-2} s^{-1}, Φ_{NPQ} of rape leaves were significantly lower in the three treatments than in the control (Fig. 4c), demonstrating that photo-protective capacity of rape grown in oxytetracycline-polluted soil was depressed. Consequently, this study showed that the light protection ability in rape leaves were significantly reduced by oxytetracycline exposure under high PAR.

Φ_{NO} is a considerable index of photo-damage. The higher value of Φ_{NO} indicates that light energy absorbed by the plant cannot be consumed completely through photochemical energy conversion and protective regulation mechanisms such as heat dissipation [34, 36]. Plants subjected to injury or continuous stress will lead to permanent damage to the plant's photosynthetic apparatus [34]. Φ_{NO} in rape leaves was higher in the three treatments than in the control under each PAR gradient. Specifically, when PAR was up to 1,500 µmol photons m^{-2} s^{-1}, amplified differences of Φ_{NO} between each oxytetracycline exposure treatment and the control were unsealed. Results showed that oxytetracycline exposure may increase the risk of injury in leaves of rape by strong light.

Subsequently, linear regression analysis was performed to characterize the relationships between the oxytetracycline concentrations and chlorophyll fluorescence parameters with amplified differences under high PAR. Fig. 5 illustrates the strong ($r^2 = 0.553$, $p<0.01$) positive correlation of oxyte-tracycline concentrations with Φ_{NO}, while weak ($r^2 = 0.435$ or lower, $p<0.01$) negative correlation of oxytetracycline concentrations with *ETR*, Φ_{PSII}, and Φ_{NPQ}.
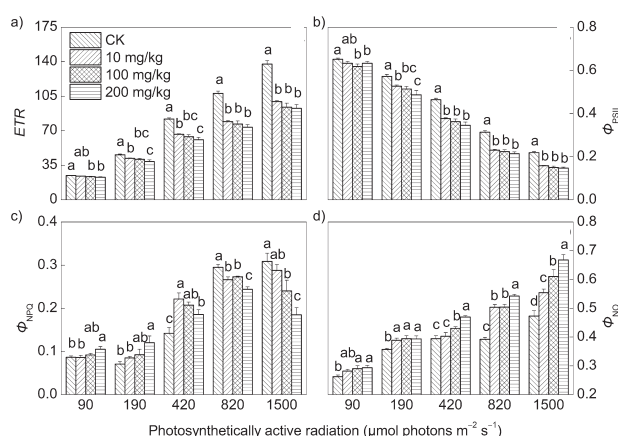

Fig. 4. Effects of oxytetracycline on electron transport rate (ETR), effective quantum yield of PSII (Φ_{PSII}), quantum yield of light induced non-photochemical fluorescence quenching (Φ_{NPQ}), and quantum yield of non-light induced non-photochemical fluorescence quenching (Φ_{NO}) of rape under different photosynthetically active radiation (mean ± SE, n = 9). Different letters above bars indicate significant differences.
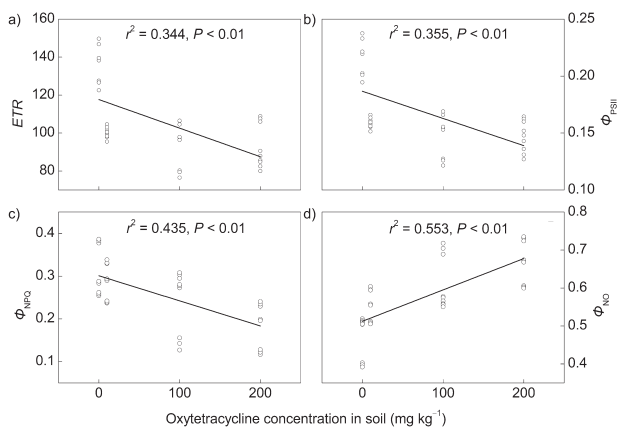
Fig. 5. Linear regression analysis of the correlations of oxy-tetracycline concentrations with electron transport rate (ETR), effective quantum yield of PSII (Φ_{PSII}), quantum yield of light induced non-photochemical fluorescence quenching (Φ_{NPQ}), and quantum yield of non-light induced non-photochemical fluorescence quenching (Φ_{NO}) of rape under 1500 µmol photons $m^{-2}\,s^{-1}$ photosynthetically active radiation ($n = 36$).

We suggest that signal detection of chlorophyll fluorescence induced by high PAR could be used to diagnosis photosynthesis capacity changes of rape threatened by oxytetracycline exposure.

Conclusions

Oxytetracycline exposure was found to inhibit the growth, leaf relative chlorophyll content, and photosynthesis capacity of rape, and also alter the energy distribution in the photosynthetic electron transport chain. The fractions of Φ_{NO} in PSII in the all-oxytetracycline treatments are especially increased with PAR increases. Photo-protective capacity and photo-damage in leaves of rape affected by oxyte-tracycline in soil to strong light were significantly changed. The present results may extend our knowledge regarding oxytetracycline effects on plant growth and development.

Acknowledgements

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