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In vitro inhibition of pigmentation and fiber development in colored cotton*

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Abstract: Colored cotton has naturally pigmented fibers. The mechanism of pigmentation in cotton fiber is not well documented. This experiment was conducted to study the effects of respiratory chain inhibitors, i.e., rotenone and thiourea, on pigmentation and fiber development in colored cotton. After 1 d post-anthesis, ovaries were harvested and developing ovules were cultured on the liquid medium containing different concentrations of rotenone and thiourea for 30 d. The results demonstrate that both respiratory inhibitors reduced fiber length and ovule development under ovule culture conditions, and the inhibition efficiency of rotenone was much higher than that of thiourea. Rotenone and thiourea also showed significant effects on fiber pigment (color) development in colored cotton. In green cotton fiber, rotenone advanced fiber pigment development by 7 d at 200 μmol/L, while thiourea inhibited fiber pigmentation at all treatment levels (400, 600, 800, 1000, and 2000 μmol/L). Both respiratory inhibitors, however, had no significant effects on pigmentation of brown cotton fibers. The activities of cytochrome c oxidase (COX) and polyphenol oxidase (PPO) decreased significantly with increasing levels of both respiratory inhibitors. It is suggested that both respiratory inhibitors have important roles in deciphering the mechanism of pigmentation and fiber development in colored cotton.

Key words: Cytochrome c oxidase, Polyphenol oxidase, Fiber length, Rotenone, Thiourea
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1 Introduction

The increasing demand for naturally pigmented cotton by fashion-conscious and ecologically minded consumers stimulated the research into the biochemistry and physiology of pigmented fibers. The cotton ovule culture technique was established four decades ago and has been applied in research related to cell biology, biochemistry, and the molecular biology of fiber development (Beasley and Ting, 1973; Beasley *et al*., 1974; Wang *et al*., 2002; Sun *et al*., 2005; Shi *et al*., 2006; Taliercio and Haigler, 2011). There are no differences between cultured ovule and field-grown plant fibers, with respect to morphological and biochemical characters, specifically fiber elongation and wall thickening (Beasley *et al*., 1974; Meinert and Delmer, 1977; Carpita and Delmer, 1981). The cotton ovule culture technique was found to be useful in exploring the biosynthesis of compounds associated with fiber pigmentation (color) in colored cotton (Schmutz *et al*., 1993; 1996).

Cotton fibers are seed trichomes and undergo some common metabolic pathways such as respiration and cellulose biosynthesis (Benedict *et al*., 1994; Jacob-Wilk *et al*., 2006), similar to other plant cells. However, the different pathways that lead to the development of pigments in colored cotton fiber are not

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yet well explored (Kohel, 1985; Murthy, 2001). This might be partially due to complex nature of fiber pigment (Schmutz *et al*., 1993; Dutt *et al*., 2004; Hua *et al*., 2007).

Several studies have shown that the pigmentation of brown and green cotton fibers might be the result of flavonoids synthesis (Hua *et al*., 2007; Xiao *et al*., 2007). The flavonoid biosynthesis pathway has been extensively investigated in many species such as *Petunia* and *Arabidopsis* (Buer and Muday, 2004). However, the mechanism of its regulation is yet not well explored. Flavonoid biosynthesis is affected by many factors such as carbohydrates (He *et al*., 2005; Hua *et al*. 2007; Huang *et al*., 2012), phytohormones (Russell and Galston, 1969; Weiss *et al*., 1990; Solfanelli *et al*., 2006), cytochrome c oxidase (COX) (Doostdar *et al*., 1995; de Vetten *et al*., 1999; Kitada *et al*., 2001), and polyphenol oxidase (PPO) (Nakayama *et al*., 2000). The deactivation of an isolated gene encoded for cytochrome b5 in *Petunia* could change the color of the flower (de Vetten *et al*., 1999). COX and PPO are important members of the respiration electron transport chain, and some chemicals such as rotenone and thiourea can inhibit their activities at different stages of respiratory pathways (Solomos and Laties, 1976; Johnson-Flanagan and Spencer, 1981). It is therefore particularly important to explore the role of different respiratory pathways involved in the pigmentation and fiber development of colored cotton. Methods and examples are needed to explore the roles of different respiratory pathways involved in pigmentation and fiber development in colored cotton. This study is an example of such a case and revealed the in vitro inhibition of respiratory pathways through respiratory inhibitors and their effects on pigmentation as well as on fiber development.

2 Materials and methods

2.1 Plant materials

Seeds of three cotton (*Gossypium hirsutum* L.) isolines, i.e., 'Xuzhou142' (white fiber cotton), 'S352' (brown fiber cotton), and 'S029' (green fiber cotton) were sown in polythene bags of size 70 mm in diameter and 100 mm in height, filled with nutrient-rich soil and placed in a greenhouse. Each polythene bag contained 2–3 seeds at the depth of 15 mm. After germination, only one healthy plant was allowed to grow in each bag, and the remaining plants were discarded. Twelve-day-old seedlings were transplanted out in the field, at a site rich in organic matter and with a pH of 6.5, at the experimental farm of the College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, China. The distances between rows and plants in the field were 0.7 and 0.4 m, respectively.

2.2 Ovule culture using respiratory inhibitors

One day post-anthesis (DPA) flowers were harvested, and ovaries were surface sterilized using 70% ethanol for 45 s, rinsed with sterile distilled water, and then immersed in 0.1% (1 g/L) mercuric chloride for 10 min. Developing ovules were carefully dissected from the ovaries under sterile conditions and immediately floated on the liquid medium containing 5 μmol/L indole-3-acetic acid (IAA) and 0.5 μmol/L gibberellic acid (GA_3) in a 100-ml flask (Beasley and Ting, 1973). The ovules were kept at 30 °C in the dark. Rotenone (Cat. No. 83-79-4, Sigma-Aldrich, Shanghai, China) was dissolved in acetone to make a 0.4 mol/L stock solution and thiourea was dissolved in distilled water to make a 1 mol/L stock solution. These stocks were sterilized by filtering through 0.22-μm organic-system and aquatic-system membranes, respectively. Both inhibitors were added to the medium before floating ovules. Rotenone levels were 5, 10, 50, 100, and 200 μmol/L, and thiourea levels were 400, 600, 800, 1000, and 2000 μmol/L. The application level of thiourea to the medium was determined by the pre-experiment, in which it was found that there was no evident effect on fiber length or ovule fresh weight at 10, 50, 100, or 200 μmol/L. Developing ovule samples were photographed using a stereo microscope (Leica MZ 95, Germany). Fiber length was measured at 30 d after culturing.

2.3 Measurement of fiber length and enzymes

The harvested ovule samples were divided into three parts. One part was boiled in water for 5 min to detach the fibers. The fibers were rinsed using distilled water and their lengths were measured using a glass ruler. The second part of the ovule sample was weighed for fresh weight assay, after the fibers were removed. The third part of the ovule was used for the

analysis of the activities of COX and PPO. For the analysis of COX activities, a 0.5-g fiber sample was removed from 30-d-old cultured ovules, ground with pre-chilled Tris-citric acid buffer (0.1 mol/L) to make a fine slurry, and was subsequently centrifuged at 20000 r/min for 30 min at 4 °C. The enzyme assay was performed according to Prasad *et al*. (1994). For the analysis of PPO activities, fibers (0.5 g) removed from 30-d cultured ovules were ground with pre-chilled phosphate buffer (50 mmol/L, pH 7.0) to make a fine slurry, and were subsequently centrifuged at 12000 r/min for 5 min at 4 °C. The enzyme assay was then performed according to Hao *et al*. (2002).

3 Results

3.1 Effects of rotenone and thiourea on fiber elongation

We initially investigated the effects of the two respiratory inhibitors on white, brown, and green cotton fiber lengths using 30-d-old floating ovule cultures. Results demonstrated no significant differences in fiber length between the control and the 5–10 μmol/L rotenone treatments in all cotton genotypes (Table 1). However, higher amounts of rotenone, particularly 200 μmol/L, showed deleterious effects on the fiber lengths of all cultured ovules irrespective of the cotton type. In contrast with rotenone, the toxic effects of thiourea on fiber elongations of white and colored cotton were relatively low. Fiber lengths of both white and colored cotton decreased with increasing concentration of thiourea ranging from 400 to 2000 μmol/L, and were 70% of the control at the highest application level; however, the average decreases of both white and colored cotton fiber lengths with thiourea application were less obvious as compared to rotenone application.

3.2 Effects of rotenone and thiourea on ovule fresh weight

We also examined the fresh weight of ovules to understand the effects of rotenone and thiourea on ovule/seed development. A drastic decrease was observed in the fresh weights of cultured ovules of white, brown, and green fiber cotton with increasing amount of rotenone (Table 2). At the highest level of rotenone (200 μmol/L), there was a marked reduction of about 70% of the fresh weights of green and brown fiber cotton ovules, in contrast to a 50% reduction of the fresh weight of white fiber cotton ovules. The fresh weight of cultured ovules, however, decreased only when thiourea was applied at 2000 μmol/L, with a 7.5% reduction in brown fiber cotton and a 3.2% reduction in green fiber cotton.

Table 1 Effects of rotenone and thiourea on white, brown, and green cotton fiber lengths after 30-d culture at different levels

Respiratory inhibitor (μmol/L)	Fiber length (mm)		
	White fiber cotton	Brown fiber cotton	Green fiber cotton
Rotenone			
0	24.07±0.51^{a}	21.97±0.45^{a}	23.23±0.93^{a}
5	23.67±1.26^{a}	22.27±0.25^{a}	22.10±0.17^{a}
10	24.67±0.29^{a}	21.33±1.89^{a}	22.83±0.29^{a}
50	20.00±0.50^{b}	16.83±0.29^{b}	16.07±1.44^{b}
100	15.00±0.50^{c}	12.83±0.76^{c}	13.23±0.25^{c}
200	12.83±0.57^{d}	9.73±0.40^{d}	9.37±0.32^{d}
Thiourea			
0	24.07±0.51^{a}	21.97±0.45^{a}	23.23±0.93^{a}
400	23.33±0.76^{a}	22.33±0.76^{a}	23.40±0.85^{a}
600	24.33±1.61^{a}	22.33±1.04^{a}	23.23±0.64^{a}
800	22.83±0.76^{ab}	21.90±0.66^{a}	22.47±1.00^{a}
1000	21.60±0.66^{bc}	19.93±0.60^{b}	19.63±0.71^{b}
2000	19.67±0.15^{c}	15.73±1.78^{c}	15.20±0.61^{c}

Results are expressed as mean±standard error (SE) (5 replicates; 5 bottles and 5 ovules taken from each sampled bottle). Different superscript letters after values in each volume indicate that the significance reached 5% possibility level (P<0.05)

3.3 Effects of rotenone and thiourea on pigmentation of colored cotton fiber

Accumulation of pigment (brown and green colors) was observed at almost same time both in vivo (field grown) around and in vitro (ovule culture). Under field conditions, pigment accumulation started around 20 and 25 DPA (Figs. 1h and 1i), while in vitro pigment accumulation was observed at 22 DPA (Fig. 1e). When rotenone concentration in medium was 0–100 μmol/L, the green color of the green cotton fiber could be visualized at 22 d after starting ovule culture. This result indicated that rotenone has no significant influence on pigment appearance up to 100 μmol/L; however, when the concentration was doubled (200 μmol/L), color development occurred almost 7 d earlier. In contrast to rotenone, when culture media were supplemented with various concentrations (400 to 2000 μmol/L) of thiourea, no pigmentation was observed until 30 d after ovule culture. Moreover, the application of either respiratory inhibitor did not cause any significant change in the pigment appearance of brown fiber cotton (Figs. 2 and 3).

Table 2 Changes of ovule fresh weights of white, brown and green fiber cotton after 30-d culture under two respiratory inhibitors rotenone and thiourea treatments at different levels

Respiratory inhibitor (μmol/L)	Ovule fresh weight (mg)		
	White fiber cotton	Brown fiber cotton	Green fiber cotton
Rotenone			
0	96.73±0.59^{a}	97.40±1.95^{a}	97.17±0.59^{a}
5	97.07±0.51^{a}	97.10±0.36^{a}	96.70±0.36^{a}
10	96.80±1.90^{a}	94.67±1.82^{b}	95.87±1.82^{b}
50	86.13±0.25^{b}	82.87±0.74^{c}	81.00±0.62^{c}
100	64.27±0.21^{c}	54.17±1.80^{d}	50.63±0.61^{d}
200	43.17±0.61^{d}	33.83±1.45^{e}	30.73±1.27^{e}
Thiourea			
0	96.73±0.59^{ab}	97.40±1.04^{ab}	97.17±0.59^{ab}
400	97.33±0.35^{a}	98.73±0.61^{a}	97.70±1.04^{a}
600	96.37±2.01^{ab}	97.50±0.82^{a}	97.57±0.91^{a}
800	96.17±0.55^{ab}	96.10±0.36^{bc}	95.83±0.42^{bc}
1000	95.20±0.56^{b}	95.63±0.95^{c}	94.70±0.75^{cd}
2000	95.07±0.55^{b}	90.10±0.56^{c}	94.00±0.62^{d}

Results are expressed as mean±standard error (SE) (5 replicates; 5 bottles and 5 ovules taken from each sampled bottle). Different superscript letters after values in each volume indicate that the significance reached 5% possibility level (P<0.05)

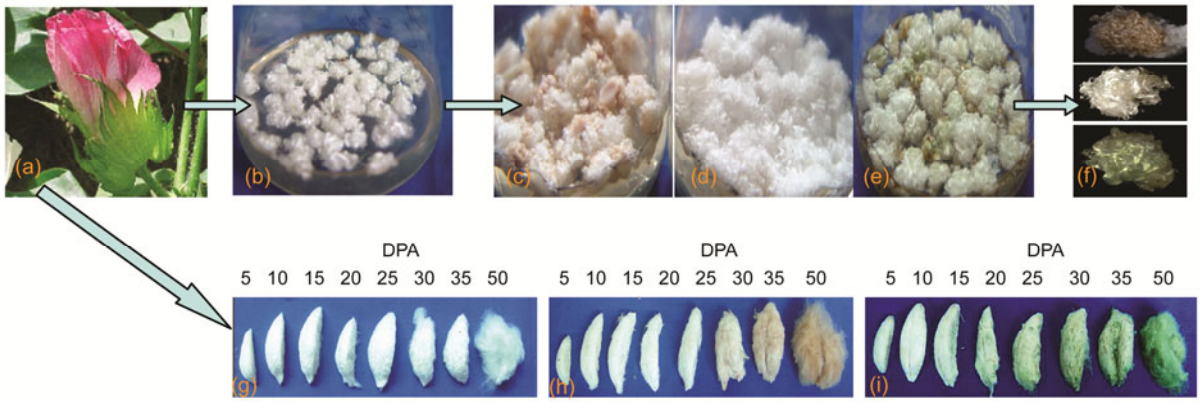

Fig. 1 Color display of white, brown, and green cotton fibers under field condition and liquid medium culture condition
(a) 1 d post-anthesis (DPA) flower; (b) 10 DPA ovules; (c) 25 DPA ovules of brown fiber cotton; (d) 25 DPA ovules of white fiber cotton; (e) 25 DPA ovules of green fiber cotton; (f) 30 DPA ovules of brown fiber cotton (panel up), white fiber cotton (panel middle), and green fiber cotton (panel down); (g) color display of white cotton fiber from 5 to 50 DPA under field condition; (h) color display of brown cotton fiber from 5 to 50 DPA under field condition; (i) color display of green cotton fiber from 5 to 50 DPA under field condition

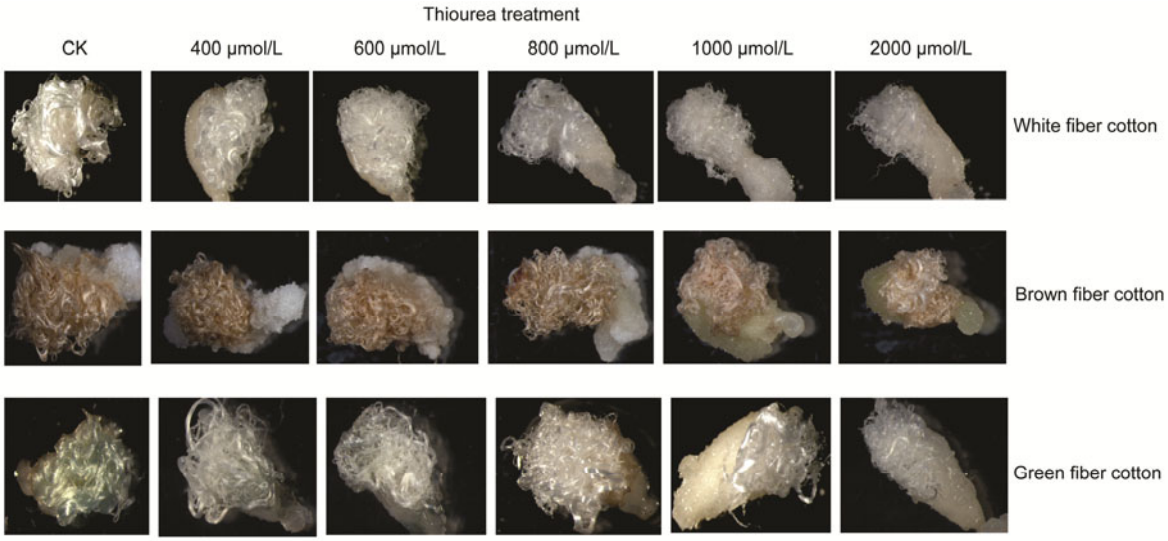

Fig. 2 Color display of white, brown, and green fiber cotton under thiourea treatment at different levels
Magnification was 0.63× under a Leica stereoscopic microscope

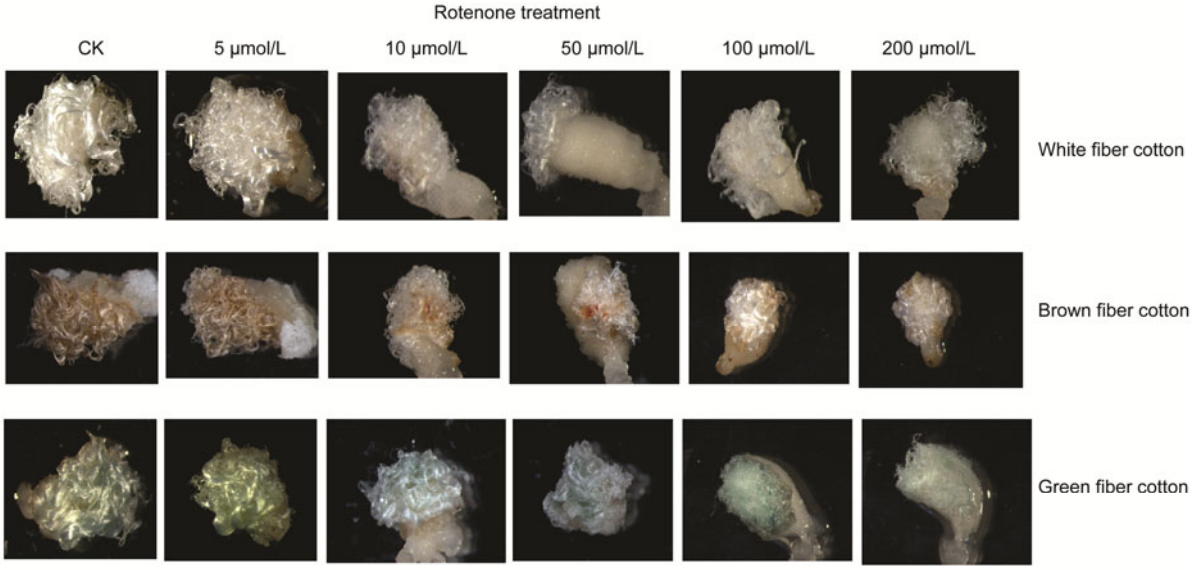

Fig. 3 Color display of white, brown, and green fiber cotton under rotenone treatment at different levels
Magnification was 0.63× under a Leica stereoscopic microscope

3.4 Effects of rotenone on COX activity and thiourea on PPO activity

It is already reported that rotenone inhibits activity of COX while thiourea affects PPO. In the present study, an effort was made to correlate these inhibitory effects with the color of the cotton fiber. The activity of COX was not affected at 5–50 μmol/L rotenone application; however, at higher levels of rotenone application (200 μmol/L), COX activity decreased in all cotton types with a maximum reduction of 90% in green cotton fiber (Table 3). The inhibitory effects of thiourea on the activity of PPO were not obvious at an application level of about 400 and 600 μmol/L in all cotton types (Table 4). A marked reduction in PPO activity was, however, found with higher concentrations (800–2000 μmol/L).

Table 3 Changes of cytochrome c oxidase (COX) activities of colored and white fiber cotton after 30-d culture under rotenone treatment at different levels

Rotenone (μmol/L)	COX activity (μmol cytochrome/(min·g FW))		
	White fiber cotton	Brown fiber cotton	Green fiber cotton
0	1.05±0.04[a]	3.64±0.14[a]	0.96±0.14[a]
5	1.03±0.01[a]	3.58±0.12[a]	1.01±0.23[a]
10	0.99±0.11[a]	3.44±0.06[a]	0.92±0.23[a]
50	0.92±0.07[a]	3.32±0.13[a]	0.88±0.09[a]
100	0.75±0.04[b]	2.43±0.11[b]	0.63±0.07[b]
200	0.14±0.06[c]	1.06±0.03[c]	0.08±0.11[c]

Results are expressed as mean±standard error (SE) (5 replicates; 5 bottles and 5 ovules taken from each sampled bottle). Different superscript letters after values in each volume indicate that the significance reached 5% possibility level (P<0.05)

Table 4 Changes of polyphenol oxidase (PPO) activities of colored and white fiber cotton after 30-d culture under thiourea treatment at different levels

Thiourea (μmol/L)	PPO activity (U/(mg·min))		
	White fiber cotton	Brown fiber cotton	Green fiber cotton
0	7.16±0.56[a]	7.08±0.79[a]	8.27±1.32[a]
400	6.33±1.35[ab]	7.37±0.23[a]	8.16±1.01[a]
600	6.54±1.34[a]	6.24±0.85[a]	7.86±1.80[ab]
800	4.80±0.18[b]	4.77±0.89[b]	6.54±1.68[b]
1 000	3.04±0.45[c]	3.41±0.75[c]	4.81±3.40[c]
2 000	2.47±0.58[c]	3.61±0.71[bc]	4.05±1.26[d]

Results are expressed as mean±standard error (SE) (5 replicates; 5 bottles and 5 ovules taken from each sampled bottle). Different superscript letters after values in each volume indicate that the significance reached 5% possibility level (P<0.05)

4 Discussion

It is well documented that rotenone can inhibit plant cell mitochondria respiration (Gutman *et al*., 1970; Johnson-Flanagan and Spencer, 1981) and the most prevalent acceptance on the activation site of rotenone is nicotinamide adenine dinucleotide (NADH) dehydrogenase. This particular enzyme will interrupt the electron transferring to coenzyme Q (CoQ) through binding dehydrogenase (Pharo *et al*., 1966; Horgan *et al*., 1968). Thus mitochondrial respiration will be negatively affected and adenosine triphosphate (ATP) production will be lowered as well (Jacobus *et al*., 1982; Li *et al*., 2003). A great deal of energy is required for the development of a zygote into an embryo, followed by maturation of the seed, and this energy is supplied by mitochondria through the catabolic process of respiration (Millar *et al*., 2011).

Similarly, fiber cell development requires a lot of energy for various metabolic processes such as cellulose synthesis and cell elongation, which are usually completed (90%) up to 30 d after anthesis (Kim and Triplett, 2001). An energy shortage at this stage can hamper the growth of the ovule as well as fiber. Rotenone, a respiratory inhibitor, either slows down or stops the activity of COX, thus interrupting normal electron transfer and production of ATP (Fig. 4). In the present study, rotenone decreased the activity of COX, resulting in decreased fiber length and ovule fresh weight in both white and colored cotton fibers. It had no effect, however, on coloration of brown or green cotton. An interesting finding is the early appearance of pigment in green cotton fiber (not found in brown cotton fiber), which suggested that high levels of rotenone might reduce its life cycle and cause it to complete its metabolism quickly to prevent damage due to toxicity.

During the terminal stage of electron transfer, there are not only COX that can activate oxygen, but also other oxidases such as PPO and alternative oxidases that can react with oxygen (Elthon and McIntosh, 1986; Tamagnone *et al*., 1998). Studies have shown that thiourea is an inhibitor of

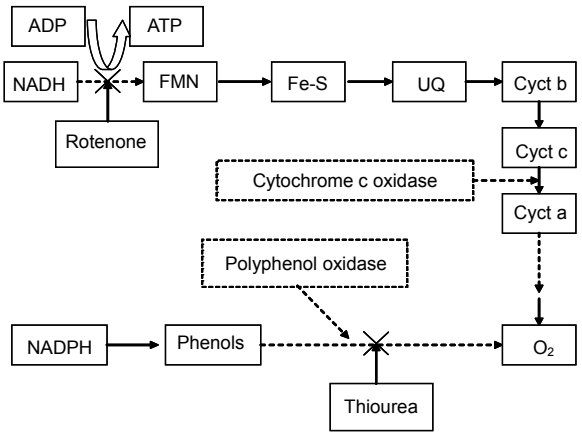

Fig. 4 Action sites of rotenone and thiourea on respiration chain

ADP: adenosine diphosphate; ATP: adenosine triphosphate; NADH: nicotinamide adenine dinucleotide; FMN: flavin mononucleotide; Fe-S: iron-sulfur proteins; UQ: ubiquinone Q; Cyct: cytochrome; NADPH: nicotinamide adenine dinucleotide phosphate

copper-containing oxidases, including PPO (Dubios and Erway, 1946). Hence the activity of PPO could be depressed by thiourea, for it contains copper ions. Compared with rotenone, thiourea had a weak toxicity effect on ovule and fiber cell growth in all cotton genotypes, but it can stop the coloration of green cotton fiber. Previous results show that the main type of pigment in green cotton fiber is flavonoids (Dutt *et al*., 2004; Hua *et al*., 2007). Flavonoid synthesis derives from a phenylpropanoid pathway with many intermediates belonging to phenyl-ramification. Improper oxidation of these intermediates may affect the synthesis of colored pigments (Winkel-Shirley, 2001). Furthermore, the phenylpropanoid pathway also results in biosynthesis of lignins (Tamagnone *et al*., 1998; Hoffmann *et al*., 2004). Therefore, if the synthesis of these two biomolecules is affected, fiber cell development can certainly be impacted. However, further investigations are required to unravel the whole mechanisms of pigment synthesis in colored cotton and its relationship with the fiber quality.

5 Conclusions

Both respiratory inhibitors can reduce fiber length and ovule development under ovule-culture conditions. The inhibition tendency of rotenone was much higher than that of thiourea. Rotenone and thiourea also showed significant effects on fiber pigment (color) development in colored cotton. In green cotton fiber, rotenone could advance the fiber pigment development by about 7 d at 200 μmol/L, while thiourea could inhibit fiber pigmentation at all treatment levels. Both respiratory inhibitors, i.e., rotenone and thiourea, had no significant effects on pigmentation of brown cotton fibers.

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