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Effects of phenolic compounds on root respiration and root activity of *Cerasus sachalinensis* Kom.

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ABSTRACT

The application of *p*-hydroxybenzoic acid and coumarin to seedlings of *Cerasus sachalinensis* changed the root respiration rate and root activity. These processes were enhanced at low concentration but decreased at high concentrations. The mixture of *p*-hydroxybenzoic acid and coumarin had additive effects on the root activity. The *p*-hydroxybenzoic acid and the mixture changed not only the biochemical respiratory pathways, but also the electron transport pathways of *C. sachalinensis* seedlings. They changed the glycolysis-tricarboxylic acid cycle (EMP-TCA) into pentose phosphate pathway (PPP) and cytochrome pathway (CP) into alternative pathway (AP). However, coumarin alone did not change the direction of respiratory pathways.

Key words: *Cerasus sachalinensis*, coumarin, phenolic compounds, *p*-hydroxybenzoic acid, respiratory pathways, root activity, root respiratory rate.

INTRODUCTION

Cerasus sachalinensis Kom. is native from the northeast mountains of China and is the most important rootstocks of cherry in these temperate areas, owing to its (i) strong cold-resistance, (ii) high survival ability and (iii) early fruiting of trees grafted on it (20). Its roots exude phenolic compounds which inhibit plant growth and development, affect permeability of membranes from root cells (18) and promoted or inhibited plant respiration (2,4,8,11,12). In sweet cherry production, the phenolic compounds exudated from the roots cause root diseases. However, the impact of phenolic compounds on respiration and physiology of cherry roots has not been reported. Therefore, this study aimed to determine (i) the effects of phenolic compounds on root respiratory metabolism and changes in root activity of *C. sachalinensis* seedlings, (ii) to investigate the rhizosphere characteristics of sweet cherries and (iii) to determine the problems caused by its continuous cropping.

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MATERIALS AND METHODS

Seeds of *Cerasus sachalinensis* Kom. were pre-soaked at 4 °C for vernalization and sown in garden soil (well-rotten manure : peat mixture 60:20:20) in a plastic tray, in greenhouse on January 2008. The chemical properties of experimental soil were: pH 7.18±0.05, electric conductivity 139.07±3.84 $\mu S \cdot cm^{-1}$, organic matter content 47.45±0.41$g \cdot kg^{-1}$, N 206.50±10.50 $mg \cdot kg^{-1}$, P 380.82±15.39 $mg \cdot kg^{-1}$, K 471.43±2.88 $mg \cdot kg^{-1}$. After emergence, the seedlings at 3 leaves stage were transplanted at one seedling per pot (13 cm ×15 cm). When the seedling roots had became strong enough at 15 leaves stage, the seedlings were treated with *p*-hydroxybenzoic acid and coumarin solutions at 50 ml per pot. Control treatment consisted in pots irrigated with 50 ml of demineralized water containing 2% ethanol. The root respiratory metabolisms and the root activity were determined one week after the treatment.

Completely randomized experimental design

The experiment consisted of two factors: (i) Phenolic compounds : 3 (*p*-hydroxybenzoic acid, coumarin and *p*-hydroxybenzoic acid + coumarin) and (ii) their concentrations: 4 (0,0.1,1.0 and 10.0 mM). The mixture was composed of *p*-hydroxybenzoic acid and coumarin in 1:1 ratio (volume). The treatments were replicated three times in a completely randomized design. The phenolic compounds were first dissolved in a small quantity of ethanol and then diluted with demineralized water to reach final concentrations of 0.1 mM, 1 mM and 10 mM. These aqueous solutions contained 2% ethanol. Demineralized water containing 2% ethanol was used as contral.

Respiration rate

Root respiration rate was measured as oxygen consumption at 25 °C using a Clark-type oxygen electrode (Hansatech Oxytherm, England) as per Karen (5) after modifications. To determine the total respiration rate, 50 mg samples of roots tissue were sliced into 1-2 mm pieces and added to 2 ml phosphate buffer (0.2 M, pH 6.8). Measurements were recorded after the oxygen consumptions was stabilized. The experiment was done in completely randomized design with three replications for each treatment. Respiration rates were expressed as $\mu mol\ O_2\text{-uptake} \cdot min^{-1} \cdot (g\ FW)^{-1}$.

Respiratory pathway

Root respiratory pathways were measured as per Yu and Pan (16). Capacity of each respiratory pathway was determined with respective inhibitor e.g. Glycolysis (EMP), tricarboxylic acid cycle (TCA) and pentose phosphate pathway (PPP) were inhibited respectively with NaF, malonic acid and Na_3PO_4 at 0.5 M. concentration. The cytochrome pathway (CP) and the alternative pathway (AP) were inhibited respectively with NaCN and salicylhydroxamic acid (SHAM) at 0.1 M. concentration. Phosphate buffer (0.2 M, pH 6.8) was used as reaction medium. Participation of each pathway in total respiration was calculated as under:

Each respiratory pathway (%) = (Total respiration – Respiration after adding inhibitor) × 100% / Total respiration .

Root activity

Root activity was measured using the TTC (triphenyltetrazolium chloride) method and expressed as the deoxidization ability ($\mu g \cdot g^{-1} \cdot h^{-1}$) (7). The 0.5 g root samples were immersed in 10 ml of a solution prepared by mixing 0.4% TTC and phosphate buffer (0.067 M, pH 7) in a ratio 1:1. Then, immersed roots were kept in the dark for 1-3 h at 37 °C. After that, 2 ml 1 M H_2SO_4 was added to stop the reaction. The immersed roots were ground and transferred into a tube with ethyl acetate cleaning solution and total 10 ml was prepared. The solution absorbance was measured at 485 nm with spectrophotometer.

Data analysis

Data were analyzed with DPS for comparisons among all treatments using Duncan's multiple range tests for mean significances (p = 0.05) and Microsoft Excel software.

RESULTS AND DISCUSSION

Roots respirations

The phenolic compounds changed the root respiration rates of *C. sachalinensis* seedlings when assayed alone and in a mixture. The respiratory rates were enhanced at low concentration but decreased at high concentrations (Fig. 1). Effects of *p*-hydroxybenzoic acid and coumarin on respiratory rates were significant at concentrations higher than 0.1 mM. At 1 mM, the *p*-hydroxybenzoic acid decreased the respiratory rate but coumarin and their mixture enhanced it. Phenolic compounds significantly reduced root respiration at concentration 10 mM (Fig. 1). Thus the effective concentrations (concentration that affects the plant respiration) of two exogenous phenolic compounds were different.

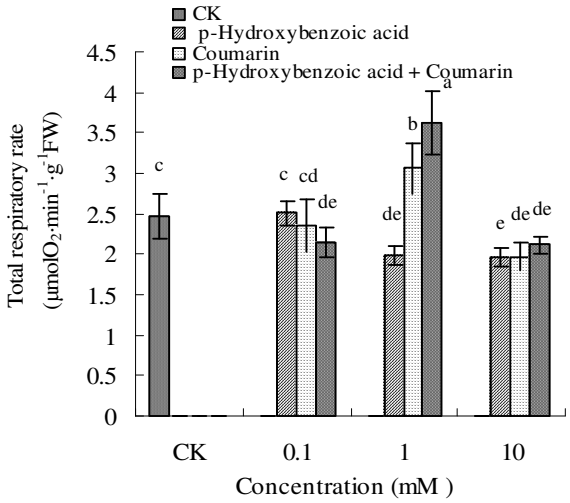

Figure 1. Effects of *p*-hydroxybenzoic acid, coumarin and their mixture under different concentrations on the root respiratory rates of *C. sachalinensis* Kom. seedlings.

Roots activities

Response observed for root activities and root respiration rates of *C. sachalinensis* Kom. seedlings had a similar trend. Root activities were enhanced at low concentrations of phenolic compounds and were reduced at higher ones. (Fig. 2). The root activities of *C. sachalinensis* seedlings were enhanced slightly (not significantly) at 0.1 mM and were significantly stimulated at 1 mM *p*-hydroxybenzoic acid and coumarin. The *p*-hydroxybenzoic acid and coumarin mixture at 0.1 mM and 1 mM concentration increased root activity suggesting that a synergistic effect occurred. On the other hand, a significant decrease in root activity was observed at 10 mM concentration and the degressive effect of mixture also showed promotion. In mixture the increase or decrease in root activity was more than the *p*-hydroxybenzoic acid or coumarin alone. The *p*-hydroxybenzoic acid and coumarin ameliorated the root activity at low concentration (1 mM) but decreased at high concentration (10 mM) and their mixture showed additive effect on root activity.

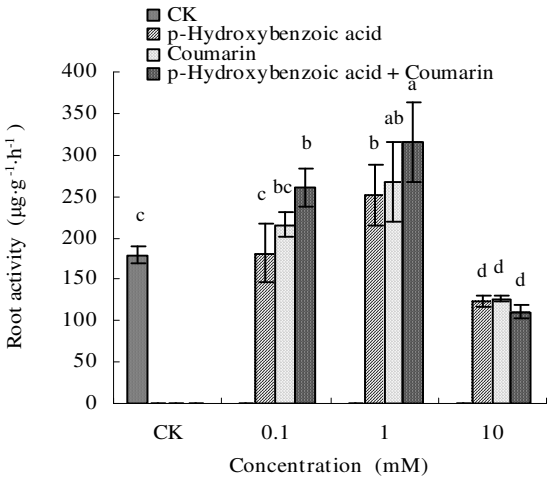

Figure 2. Effects of *p*-hydroxybenzoic acid, coumarin and their mixture under different concentrations on the root activities of *C. sachalinensis* Kom. seedlings.

Roots biochemical respiratory pathways

In normal growth conditions, EMP-TCA (Glycolysis-Tricarboxylic acid) were dominant respiratory pathways in roots of *C. sachalinensis* seedlings and accounted for 65.42% of total biochemical respiratory rate. PPP (Pentose phosphate pathway) pathway accounted for only 40.87% (Fig. 3).

In roots treated with *p*-hydroxybenzoic acid, the EMP pathway percentage rose significantly from 22.85% to 51.73% at 0.1 mM concentration, while proportion entered into TCA sharply dropped from 65.42% to 30.19% (Fig. 3). PPP pathway slightly rose to 2.14%. Percentage of EMP pathway dropped sharply and TCA proportion declined, when concentrations were increased to 1 mM and 10 mM. Consequently, PPP pathway became

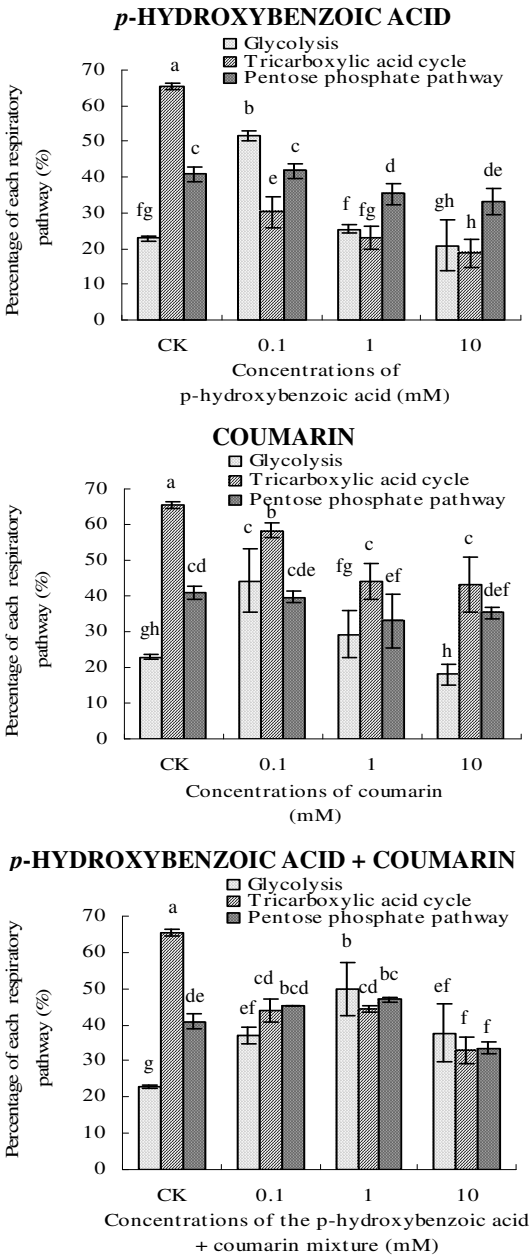

Figure 3. Effects of *p*-hydroxybenzoic acid, coumarin and their mixture under different concentrations on the root biochemical respiratory pathways of *C. sachalinensis* seedlings

the leading respiratory way. Similar trends of respiratory pathway at 1 mM and 10 mM concentrations were seen and the PPP pathway replaced the EMP-TCA pathways. The proportions of PPP both at 1 mM and 10 mM concentrations were significantly higher than EMP-TCA pathways. It is obvious that p-hydroxybenzoic acid influenced either the proportions of root respiratory rates or the biochemical respiratory pathways of *C. sachalinensis* seedlings.

Although the leading respiratory pathways of *C. sachalinensis* seedlings were not altered by coumarin but its each concentration changed the proportion of each respiratory pathway than control (Fig. 3). Application of 0.1 mM coumarin, significantly increased the percentage of EMP pathway from 22.85% to 44.31% and the proportion entered into TCA fell significantly from 65.42% to 58.22%. However the PPP pathway did not change. The respiratory pathways also changed similarly at increased concentrations of 1 mM and 10 mM and the percentage of each respiratory pathway followed the order TCA > PPP > EMP. The overall trend showed that the EMP-TCA path was always leading and followed by PPP path. Although, the coumarin influenced only the proportion of each respiratory path but did not change their directions.

The application of mixture of *p*-hydroxybenzoic acid and coumarin had different effects on the root respiratory pathways (Fig. 3). When roots were treated by 0.1 mM solution, percentage of EMP path was significantly higher than control. Proportion entered into TCA was accordingly reduced significantly and PPP path was slightly improved. Ultimately, PPP became the leading one among the three pathways. Percentage of EMP path rose to 49.92% (significantly over the control) at the increased concentration of 1 mM. TCA and PPP proportion slightly changed and both were lower than the EMP. Three pathways proved relatively equal. Percentage of each respiratory pathway decreased at 10 mM, but the total trend of respiratory pathway was identical to 1 mM concentration and the effective entrance of EMP into TCA was decreased and both followed the trend EMP > PPP > TCA. The mixture of *p*-hydroxybenzoic acid and coumarin also changed the root biochemical respiratory pathways of *C. sachalinensis* seedlings.

Roots Electron transport pathways

When grown in normal conditions, the electron transport pathways in the roots of *C. sachalinensis* seedlings gave priority to CP (Cytochrome Pathway) which accounted for 43.15% of total respiration rate. The AP (Alternate Pathway) only covered 13.39% (Fig. 4).

The p-hydroxybenzoic acid significantly increased percentage of participation of AP at 0.1 mM concentration when compared to control while percentage of CP remained constant (Fig. 4). At 0.1 mM, percentage of AP did not differ significantly from CP. AP (%) increased at 1 mM and then declined at 10 mM, while, CP (%) decreased with the increasing concentrations. The AP became the major respiratory pathway at concentrations > 0.1 mM and was maximum at 1 mM.

The coumarin significantly increased the percentage of participation of AP at 0.1 mM concentration than control, while percentage of CP remained constant (Fig. 4). At 0.1 mM, percentage of AP was significantly lower than CP. Percentage of CP (%) reached maximum at 1 mM and then decreased. Percentage of participation of AP in total respiration followed the same trend as CP. Nevertheless, CP was the predominant functioning respiratory pathway at all concentrations of coumarin assayed.

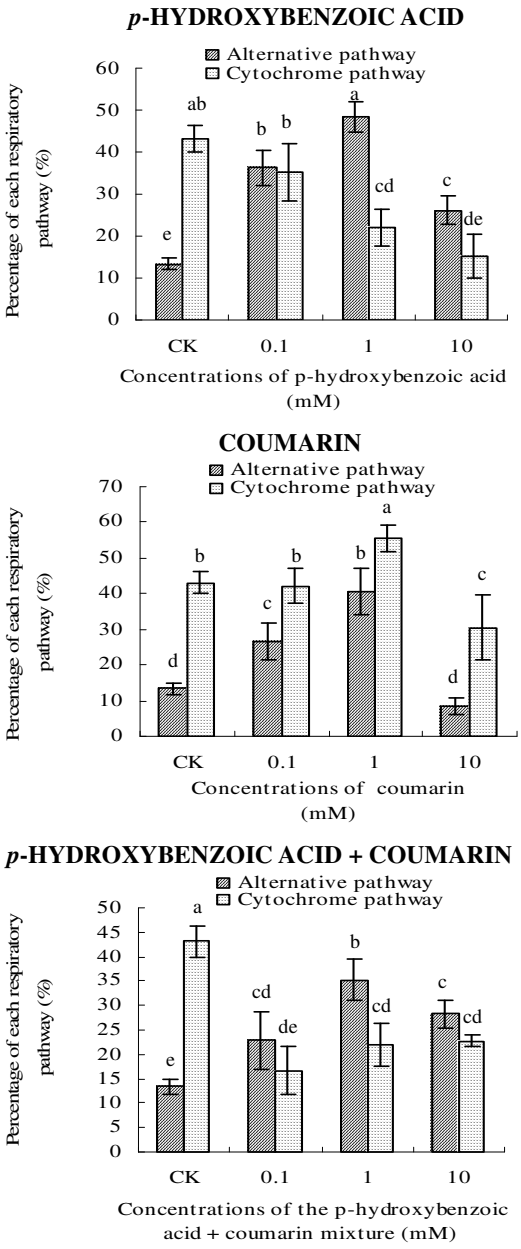

Figure 4. Effects of *p*-hydroxybenzoic acid, coumarin and their mixture under different concentrations on the electron transport pathways of *C. sachalinensis* seedlings

The mixture of phenolic compounds significantly changed percentages of participation of electron transport pathways respect to control (Fig. 4). Percentage of CP was significantly reduced by the mixture, while that of AP was increased. Highest AP participation was observed at 1 mM concentration. For those roots treated with the mixture, percentage of AP was higher than that observed for CP, only at concentration 1 mM.

Under normal conditions, the root activity increased with the increase in respiration rate (6). The root activity increased because respiration provided the energy for root metabolism and active absorption. The main principle of TTC method to determine root activity was to measure the respiratory-related dehydrogenase such as succinate dehydrogenase. The reduced amount of TTC indicated the dehydrogenase activity of root tissue (7), hence, the deoxidized TTC capacity and respiration ability are related to each other (19). In this experiment, positive correlation between the root activity and root respiratory rate of *C. sachalinensis* seedlings treated by coumarin and the mixture was greatly significant ($r = 0.965$), but negative correlation was not significant, when treated by *p*-hydroxybenzoic acid ($r = -0.313$). Perhaps, because the *p*-hydroxybenzoic acid solution was acidic. When *p*-hydroxybenzoic acid was dissolved in soil, the root mitochondria were damaged under acidic conditions (10,14). Energies were in short supply caused by oxidative phosphorylation uncoupled. Instead, coumarin was slightly acidic and it would be weakened further after the two phenolic compounds were mixed. That is why the roots were not too sensitive to coumarin and the mixture. Beside, it could be considered an adaptable response to environmental stress that root activity increases with the root respiration rate. Previous study indicated that allelochemicals influenced the plant respiration in two ways: restraining the electron transport of mitochondria and restraining the process of oxidative phosphorylation (1). Phenolic compounds at 10 mM could badly reduce the root functions of *C. sachalinensis* seedlings, which decreased the respiration rate and production of ATP that could not fulfil the roots requirements. That is why the root activity decreased when the respiratory rate declined at high concentration. Therefore one of the reasons, why trees decline rapidly after planting in place of many years old trees or continuously cropping, due to accumulated phenolic compounds which injured the respiratory system of fruit tree roots.

The effects of phenolic compounds on plant root respiratory pathways yet has not been studied in-depth. However, correlative researches indicated that the exudates of sorgo roots could held back the electron transport between the cytochrome complexes *b* and *c* in the mitochondria of maize and soybean leaves which prevented from the cytochrome pathway (3). Wu *et al.* (15) found that the proportion of TCA in rice leaves and roots was reduced, while PPP increased, when treated by methyl jasmonate, but there were no changes in the respiratory electron transport chain pathway. Zhang (17) proved the proportion of EMP, TCA and PPP pathway of biochemical respiration and alternative pathway of electron transport chain were all lower than the control, while the proportion of cytochrome pathway was firstly higher and then became lower than control, when *Pyrus pyrifolia* was treated with salicylic acid. It was obvious that different secondary metabolites had different effects on respiratory pathways of plant roots. This experiment testified the *p*-hydroxybenzoic acid and the mixture of *p*-hydroxybenzoic acid and coumarin could change not only the biochemical respiratory pathways but also the pathway of respiratory electron transport chain in the *C. sachalinensis* Kom. seedlings. They turned the EMP-TCA into PPP of the biochemical respiratory pathway and CP into

AP of the electron transport pathway. It explained that the PPP pathway and the alternative pathway were enhanced when plants suffered from diseases, injuries and stresses (13). However, coumarin could not change any respiratory pathway of *C. sachalinensis* seedlings. The phenolic compounds might alter the respiratory pathway ultimately through influencing the electron transport in root mitochondria.

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