Pak. J. Bot., 45(1): 135-139, 2013.

TOXICITY INDUCED BY SOLANAPYRONE A IN CHICKPEA SHOOTS AND ITS METABOLISM THROUGH GLUTATHIONE/ GLUTATHIONE-S-TRANSFERASE SYSTEM

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Abstract

Solanapyrone A and C were isolated from a Pakistani isolate of *Ascochyta rabiei*, Pk-1. Two experiments were conducted to investigate the phytotoxic effects of the most potent toxin, solanapyrone A on chickpea cultivars and its subsequent detoxification through glutathion/glutathion-s-transferase(GST) system. When the shoots of cultivars were fed solanapyrone A, symptoms mimicking to Ascochyta blight appeared and extent of manifestation of symptoms varied with the cultivar. In the first experiment, the effect of three different plant ages of 2 cultivars with different levels of resistance to toxin was determined in terms of GST activity unit. GST activity in Balkasar-2000 (a resistant cultivar) increased 1.92 times, 1.72 and 1.65 times in two-week-old seedling, eight-week-old and adult plants (all treated) respectively as compared to their respective controls. In the highly susceptible cultivar, AUG-424, a slight increase (1.14 times) over control was noticed in GST activity at all the three ages. In the second experiment, where shoots of three cultivars were tested against 2 doses of the toxin, an increase in GST activity in Noor-91 (a moderately susceptible cultivar) and AUG-424 was significantly less than resistant cultivar, Balkasar-2000 showing direct relationship between resistance and activity of the enzyme. It may be concluded that it is a reason for difference in response of cultivars to the disease.

Introduction

Ascochyta blight, caused by *Ascochyta rabiei*, is a destructive disease of chickpea (*Cicer arietinum* L). The toxins solanopyrone A, B and C produced by the fungus were found to be involved in its pathogenicity and the symptoms of blight were consistent with toxin production by the pathogen (Hamid & Strange, 2000). Application of purified solanopyrones to chickpea leaves produced visible symptoms in 24 h, followed by contraction of protoplasts of epidermal, palisade, and spongy parenchyma cells (Hohl *et al.,* 1991). Although, all the solanapyrones have been extracted from the culture, only solanapyrone C has been claimed to be found in infected plants (Shahid & Riazuddin, 1998). Other workers have failed to find any of the three compounds which may indicate the metabolism of the solanapyrones through some mechanisms by host tissues of infected plants. There are some examples of metabolism of phytotoxins by various host plants. For instance, a detoxification mechanism has been found in *Pinus sylvestris* in which cell cultures metabolize fomannoxin to fomannoxin alcohol and subsequently to fomannoxin acid β-glucoside both of which are less toxic than famannoxin (Zweimuller *et al*., 1997).

Enhanced activity of defense-related enzymes is an active metabolic initiative which activates plant defense responses (Dixon *et al.*, 1998). Increase in activity of different defense-related enzymes in infected resistant cultivars of chickpea has been reported by numerous researcher (Hamid, & Strange, 2000; Vir & Grewal, 1975; Nehra *et al*., 1994; Vogelsang *et al*., 1994).The glutathione/glutathione-S-transferase system is one of the various active metabolic initiatives by which plants detoxify xenobiotics (Lamoureux *et al*., 1991; Coleman *et al*., 1997). Glutathione (GSH) is a tripeptide L-γ-glutamyl-L-cysteinylglycine found in many plant, bacterial and mammalian cells. Glutathione-S-transferases (GSTs) are a family of dimeric multifunctional enzymes (Dixon *et al.*, 1998). In plants, they are involved in endogenous functions such as cellular protection against pathogen attack and oxidative damage (Marrs, 1996). GSTs play a role in the detoxification of a diverse range of xenobiotics such as herbicides and toxins in plant (Coleman *et al*., 1997). In chickpea, GSTs were shown to covalently link solanapyrone A to GSH to form less reactive and more polar glutathione-S-conjugates in the cytoplasm (Hamid & Strange, 2000).

Since solanapyrones are involved in pathogenicity and the above precedents for the metabolism of toxins by host tissues of plants may explain why some of these compounds cannot be extracted from infected (chickpea) plants. Therefore it is important to investigate solanopyrones production by Pakistani isolate, their toxic effects on different cultivars, metabolism and the effect of solanapyrone A on the activity of defense related enzyme like GST. Accordingly, experiments were conducted to determine the toxicity of the most potent toxin solanapyrone A, its reaction with glutathione and activity of GST in chickpea cultivars in order to determine the impact of age of cultivars and differential doses of solanapyrone A on GST activity.

Materials and Methods

Growth of plants: Seedlings of the resistant and the highly susceptible cultivars of our epidemiological trials (Riaz *et al*., 2006) Balkasar-2000 and AUG-424, respectively, were grown in greenhouse at 25± 2°C in John Innes No.2 compost in plastic pots (13 cm dia.). As shoots of these cultivars were to be tested at three physiological stages i.e., 2, 8 and 14 weeks after sowing,

representing the seedling, vegetatively mature and podding stage so seeds were sown at 6 week intervals for 2nd and 3rd times. Plants of another Kabuli moderately susceptible cultivar Noor-91 were grown for only 2 weeks to use their shoots in enzyme assay against differential doses of solanapyrone A.

Detection, isolation and recognition of the solanapyrone toxins: The Pakistani isolate of *A. rabiei,* Pk-1, was used for the detection and isolation of the host-selective solanapyrones. The isolate was cultured and the toxins were separated following the method of Hamid and Strange, 2000. Fractions containing pure solanapyrones were recognized by their characteristic U.V. spectra (Ichihara *et al*., 1983).

HPLC of solanapyrones: Toxin samples of Pk-1 and reference standards (kindly provided by Dr. Richard N. Strange) were separated on a Philips HPLC equipped with a diode array detector according to the method of Chen *et al*., 1991 except with the little modification in mobile phase. The solanapyrones were recognized by their retention times and UV spectra at λ_{max} = 327; 303 and 320 nm which were compared with those of authentic samples.

As objectives of the study were to test toxigenicity of the most toxic of the solanapyrones, the solanapyrone A, and determine its reaction with glutathione for GST activity, only solanapyrone A was quantified. For quantification, chromatograms were abstracted from the three dimensional chromascans (absorption x wavelength x time) at λ_{max} = 327 nm and the area under the peak was compared with that of standard solution of the authentic compound.

Solanapyrone A uptake and symptoms development: To determine the effects of incubating solanapyrone A with shoots of different plant ages of two cultivars of different levels of resistance/susceptibility, solanapyrone A (90.6 µg in 2 µ1 ethanol) was vortexed with 2 ml water in polypropylene centrifuge tubes [115 mm x 30 mm (diam.)] to give a 150 µM solution. Four shoots (0.75g each) of each chickpea cultivar viz., Balkasar-2000 and AUG-424: 2-, 8- and 14- weeks-old) were placed in the tubes and were allowed to take up 1.5 ml of the solution (= 68µg solanapyrone A) under greenhouse conditions (23 ± 2°C: 5 to 6 h incubation period). After uptake of the toxin, shoots were transferred to tubes containing distilled water (25 ml) and incubated for a further 96 h under the same conditions. The water level was maintained throughout the incubation period. Shoots incubated in distilled water without solanapyrone A served as controls. After the incubation period, symptoms were observed on all shoots of both the cultivars and two-week old plant shoots were also photographed.

To determine the effect of two different concentrations of solanapyrone A on the cultivars, 4 shoots each (0.75 g) of 2 weeks-old plants of Balkasar-2000, Noor-91 and AUG-424 were placed in the tubes and were allowed to take up 1.5 ml each of the two solutions containing 60.4 µg and 20.1 µg in 2 µ1 ethanol (giving 100 µM and 33 µM solutions) vortexed with 2 ml of water. This made actual amounts taken equal to 45.3 µg and 15.1 µg, respectively. Both experiments were performed twice and then shoots were subjected to extraction of filtrate as given below.

Extraction and measurement of GST activity in chickpea shoots: After the incubation period, the parts of shoots covered by solanapyrone A solution or water were discarded and the entire remaining material was used for the estimation of GST activity. GST activity was determined in shoots treated with solanapyrone A by modification of the method of Hunaiti and Ali, 1990. The whole procedure was repeated for the experiment of differential doses of solanapyrone A.

Results

HPLC: There was a clear evidence for the presence of solanapyrone A and C in extracts of aggressive isolate Pk-1 as manifested by HPLC retention characteristics and UV maxima for diode array spectra (not shown here). The reverse phase HPLC retention characteristics of extracts from 12 –day-old cultures of Pk-1 contained two major components (retention time 14.856 and 18.137 minutes). The UV maxima for the diode array spectra for both components when compared with those of reference standards, confirmed the presence of both toxins. There was no evidence of presence of solanapyrone B in extracts of the culture.

Solanapyrone A uptake and symptoms development: When shoots of cultivars Balkasar-2000 and AUG-424 were allowed to take up 68µg of solanapyrone A, symptoms mimicking the blight were observed. Extent of manifestation of symptoms after taking up the dose varied and depended on the cultivar. On Balkasar-2000, symptoms were characterized by epinasty of petiole and main stem with the light pale color of leaflets in two-week old plants (Fig. 1). However, necrosis of the tissues and breakage of stem was not noticed in seedling shoots. Symptoms were more severe on adult plant shoots. Stems became shriveled and brown with flame-shaped chlorotic areas on leaflets. In eight-week old plant shoots, the symptoms were closer to 2-week old seedlings. In case of AUG-424, main stem of 2-week old seedlings could not maintain turgidity and broke (Fig. 2). Similar symptoms were observed in the shoots of 8- and 14-week old plants of this cultivar. In the second experiment, reaction (symptom development) of Noor-91 and AUG-424 to lower dose (15.1µg) of the toxin was not significantly different from each other and leaflets of both cultivars developed small chlorotic patches while shoots of Balkasar-2000 remained unaffected. However, at 45.3µg dose, bleaching at the base of stems of Noor-91 and AUG-424 occurred with more severe in AUG-424 resulting in their breakage. Shoots of Balkasar-2000 turned light pale brown with the main stem desiccated without breakage. Plants in control of both the experiments were found healthy.

Fig. 1. Epinasty of petioles and young branches of shoot of a resistant chickpea cultivar, Balkasar-2000, after taking up 68µg of solanapyrone A and incubating for further 96 h in water.

Fig. 2. Breakage of shoot of a susceptible chickpea cultivar, AUG-424, just below the upper most leaf after taking up 68µg of solanapyrone A and incubating for further 96 h in water.

Effect of plant age and toxin concentrations on GST activity: GST activity in shoots of 2-week, 8-week-old and adult plants after taking up the above amount of solanapyrone A was found to be the highest in 2-week-old seedling followed by 8-week-old plants and adult plants in the order in both cultivars after 96 hours of incubation (Table 1). There was a highly significant (p0.001) difference in GST activity of controls as well as in the treated shoots of both the cultivars at all three ages tested. GST activity in Balkasar-2000 increased 1.92, 1.72 and 1.65 times in two-week-old seedling, eight-week-old and adult plants (treated) respectively as compared to their respective controls. In the susceptible cultivar, AUG-424, the same slight increase (1.14 times) over the control was noticed in GST activity at all the 3 ages.

In the experiment of effects of two concentrations of solanapyrone A on activity of GST, shoots of cultivars Balkasar-2000, Noor-91 and AUG-424 were allowed to take up two doses i.e.,45.3 µg and 15.1 µg of solanapyrone A per shoot. Significant increase of 1.68 times and 1.27 times was observed in Balkasar-2000 after taking up 45.3 µg and 15.1 µg respectively (Table 2). In case of Noor-91, this activity increased from 48.1 ± 4.1 units (in control) to 60.9 ± 5.9 and 54.4 ± 6.1 units in shoots treated with 45.3 µg and 15.1 µg, respectively. While in AUG-424, GST activity raised only 1.13 times and 1.06 times after taking up 45.3 µg and 15.1 µg of the toxin, respectively showing its negligible ability to detoxify the toxin.

Table 1. GST activity (units) of chickpea cultivars g^{-1} fresh weight of shoots (at 340nm) of 2-week-old, 8-week-old and adult plants (14-week-old) after taking up 68 µg of solanapyrone A.

Cultivar	2-week-old Plants		8-week-old plants		Adult plants (14-week-old)	
	Control	Treated	Control	Treated	Control	Treated
Balkasar-2000	66.8 ± 1.6 c*	128.6 ± 4.6 f	64.4 ± 2.0 c	110.8 ± 3.9 e	58.3 ± 2.2 bc	96.2 ± 3.5 d
AUG-424	41.8 ± 1.4 a	48.0 ± 1.3ab	38.7 ± 1.9 a	44.5 ± 1.9 a	35.8 ± 1.7 a	40.8 ± 1.9 a

Each value is the mean of four replications

*Any two means not sharing a common letter differ significantly at 5% level of significance

Table 2. GST activity (units) of chickpea cultivars g^{-1}fresh weight of shoots (at 340nm) of two-week-old plants in control (untreated) and treated with two concentrations of solanapyrone A.

Cultivar	Control	Treated with Solanapyrone A	
		45.3 µg	15.1 µg
Balkasar-2000	67.8 ± 6.9 c*	114.0 ± 10.30 e	86.2 ± 7.8 d
Noor-91	48.1 ± 4.1 ab	60.9 ± 5.9 bc	54.4 ± 6.1 abc
AUG-424	42.4 ± 3.8 a	48.2 ± 5.7 ab	45.2 ± 4.9 a

Each value is the mean of four replications

* Any two means not sharing a common letter differ significantly at 5% level of significance

Discussion

Absence of solanapyrone B in our isolate was in agreement with the findings of several researchers (Alam *et al*., 1989; Latif *et al.,*1993 and Chen and Strange, 1994) as they observed the production of solanapyrones A and C among most of their isolates of *A.rabiei*. In contrast, Hohl *et al.*, (1991) and Chen & Strange (1991) observed the presence of solanapyrones A, B and C and concluded that the components produced were dependent on the basal medium used. In the work of Hohl *et al*., (1991). Solanapyrone B was found to be the major toxin in nine isolates while Shahid and Riazuddin, (1998) reported only solanapyrone C in their samples. Kaur (1995) observed only solanapyrone A in spore germination fluid of *A. rabiei*. Observation of Kaur (1995) that solanapyrone A can be produced in the absence of solanapyrone B may indicate that solanapyrone A is the immediate precursor for solanapyrone B.

In both experiments, difference in symptoms expressed after taking up different doses of solanapyrone A revealed that although genetics of the cultivars had an important role but the amount of the toxin fed to shoots was also very crucial. The symptoms illustrated in Figs.1 and 2 were characteristic symptoms of blight. Breakage of stem (Fig. 2) may be explained by the loss of turgor of parenchymatous cells surrounding the stele as a consequence of the attack by the toxin on their plasma membranes. Such stem would have only the inadequate support of their steles and not the additional strength imparted by the turgor of the surrounding cells (Hamid & Strange, 2000). From the results, it was obvious that plant age could only influence the toxin metabolism when the subjected cultivar was having the genes conferring resistance. In the second experiment, reaction of susceptible cultivars to solanapyrone A revealed that a specific dose of toxin was necessary to incite severe disease symptoms and below that level, even the susceptible cultivars were able to withstand the toxin. Comparison of relative sensitivities (symptoms development) of the cultivars to solanapyrone A with their disease ratings to Ascochyta blight (Riaz *et al*., 2006) showed that those that were more sensitive , such as Noor-91 and AUG-424 , were also more susceptible to the disease, while less sensitive cv. like Balkasar-2000 was also resistant to the disease. One reason for their differential sensitivity of cultivars to the toxin could lie in their ability to detoxify the compound through glutathione/ glutathione-s-transferase system. Our results corroborate the findings of Hamid and Strange (2000) showing the positive correlations between sensitivity to toxins and severity of disease symptoms.

A significant increase in GST activity in Balkasar-2000 but a non significant (p<0.001) increase was observed in AUG-424 at three different ages, indicating that the increase in GST may depend upon their level of resistance/susceptibility. A similar and slight increase in GST in case of AUG-424 indicated that age was not a significant contributing factor towards increase in GST activity in susceptible cultivars. These results indicated a positive relationship between GST activity units and resistance of cultivars and negative between age and GST activity units in resistant cultivars. These findings suggest that the defense mechanism in plants weakened with an increase in age and adult plants were more vulnerable to Ascochyta blight than young ones. These results are in agreement with the findings of (Marrs, 1996; Coleman *et al.*, 1997; Hamid & Strange, 2000) also reported more increase in reduced glutathione and GST activity in a resistant variety INRAT-88 as compared to AUG-424.

Comparison of GST activity at three physiological stages of Balkasar-2000 with its disease rating to Ascochyta blight in glasshouse trials carried out by Riaz *et al*., (2006) showed that the stage which was more sensitive to the disease was also having less GST activity. It confirmed the results of our glass house study reporting a decrease in resistance in the plants against the disease with an increase in age. Studies on GSH/glutathione-S-transferase system status with advancing age are few in plants but to date there does appear to be a negative correlation between age and GST activity (Coleman *et al.*, 1997).

In the cultivar AUG-424, little and almost similar increase in GST activity after taking up differential doses of the toxin again revealed its negligible ability to detoxify the toxin. Results showed a positive and significant correlation between toxin concentrations and GST activity in the resistant cultivar. This correlation was found to be very strong (99.5%) particularly in shoots of two-week old plants. Although an increase in GST activity was noted in Noor-91 and AUG-424, it was significantly (p<0.001) less than Balkasar-2000 showing a direct relationship between the resistance and the activity of the enzyme. An increase in GST activity in chickpea plants after taking up oxidiazon was previously reported by Hunaiti & Ali (1990).

In this study the reason for difference in sensitivity of cultivars at two different doses of solanapyrone A and also at three different plant ages was sought. The glutathione / glutathione-S-transferase system is one of the various mechanisms by which plants detoxify xenobiotics (Coleman *et al.*, 1997; Lamoureux, 1991). Marrs (1996) found that glutathione was a significant component of the antioxidant defenses, and a highly potent antioxidant and antitoxin in its own right. GSTs catalyze the conjugation of the thiol group of glutathione to various endogenous or exogenous substrates to form polar and nontoxic peptide conjugates (Coleman *et al.*, 1997; Marrs, 1996). Such conjugates are more hydrophilic than the original compounds, which decrease their ability to integrate into biological membranes and restrict their accumulation within cells and tissues (Coleman *et al.*, 1997). In this study some evidence relating to the detoxification of solanapyrone A by this mechanism was obtained through the formation of an adduct of GSH and 1-chloro-2, 4-dinitrobenzene (CDNB). Demonstration of the conjugate *in planta* may prove difficult, however, since glutathione conjugates of toxins are often rapidly metabolized (Marrs, 1996). These studies will be extended to other cultivars differing in resistance to establish responses of glutathione / GST to various doses of soalanapyrone A and metabolism of the toxin through other specific mechanisms.

Acknowledgements

This research work was funded by Higher Education Commission, Pakistan under International Research Support Initiative Programme enabling first author to carry out the study at Birkbeck, University of London. First author wishes to thank the commission for funding. We thank Dr. Richard Strange for the provision of reference standards of toxins. Technical assistance for HPLC at Birkbeck, University of London provided by Jason Taylor is also appreciated.

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(Received for publication 30 January 2011)