Glutathione and Cysteine Accumulation in Leaves of a Highly-Virus-Tolerant Cultivar of Zucchini (*Cucurbita Pepo* L.) in Response to Drought and Recovery

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บทคัดย่อ

การศึกษาการตอบสนองด้านการกำจัดอนุมูลอิสระของแตงซูกินี ชื่อว่า ควินี สายพันธุ์ทนทานต่อการเกิดโรคจากไวรัส เมื่อได้รับภาวะขาดน้ำและหลังจากกลับมาได้รับน้ำปกติ การให้ภาวะขาดน้ำทำโดยการงดให้น้ำต้นพืช 10 วันในช่วงระยะที่มีการเจริญของใบอย่างเต็มที่ หรือระยะก่อนออกดอก และชุดควบคุมเป็นพืชที่ได้รับน้ำปกติตลอดการทดลอง การวิเคราะห์การสะสมกลูตาไธโอนและซิสเทอีน ทำโดยการเก็บตัวอย่างใบอ่อนที่เจริญเต็มที่จากพืชที่ได้รับภาวะขาดน้ำเป็นเวลา 10 วันและหลังจากกลับมาได้น้ำปกติเป็นเวลา 14 วัน ผลการศึกษาพบว่าภาวะขาดน้ำส่งผลให้พืชมีการสะสมกลูตาไธโอนทั้งหมด เพิ่มขึ้นร้อยละ 153.29 เมื่อเทียบกับชุดควบคุม แต่ไม่มีผลต่อความเข้มข้นของซิสเทอีนทั้งหมดและออกซิไดซ์ซิสเทอีน ในต้นแตงอายุ 5 สัปดาห์ที่ปลูกในภาวะขาดน้ำและกลับมาได้รับน้ำเป็นเวลา 7 วัน มีลักษณะต้นพืชที่แตกต่างจากชุดควบคุม ได้แก่ ปลายยอดพืชไม่เจริญ ลำต้นมีปล้องสั้น ใบพืชมีก้านใบสั้นและแผ่นใบขนาดเล็กไม่แผ่ขยาย และอาจพบใบพืชมีสีซีดเหลือง และเมื่อต้นแตงมีอายุ 6 สัปดาห์จะเห็นได้ว่าต้นที่ได้รับภาวะขาดน้ำมาก่อนมีการสร้างดอกและผลอ่อน จำนวนน้อยกว่าชุดควบคุม

จากการวิเคราะห์ความสัมพันธ์ของกลูตาไธโอนและสารตั้งต้นซิสเทอีน พบว่าปริมาณซิสเทอีนทั้งหมดและกลูตาไธโอนทั้งหมดมีความสัมพันธ์กันเชิงบวก ในขณะที่การเพิ่มขึ้นของกลูตาไธโอนทั้งหมดทำให้เกิดออกซิไดซ์กลูตาไธโอนลดลง ที่ค่าความสัมพันธ์ -0.717 ระดับความเชื่อมั่นร้อยละ 95 จากผลการศึกษาเสนอได้ว่าการสร้างและสะสมสารตั้งต้นซิสเทอีนน่าจะมีความเกี่ยวข้องกับการสร้างกลูตาไธโอน ซึ่งจะถูกนำไปใช้เป็นตัวช่วยในการทำลายอนุมูลอิสระที่พืชสร้างขึ้นภายใต้ภาวะขาดน้ำ และกล่าวได้ว่าพืชที่ปลูกในภาวะขาดน้ำในช่วงก่อนการออกดอกมีผลกระทบอย่างยิ่ง ต่อการเจริญเติบโตของส่วนยอดพืชและอวัยวะสำหรับการสืบพันธุ์ เช่น ดอกและผลด้วย

คำสำคัญ : กลูตาไธโอน, ซิสเทอีน, ภาวะขาดน้ำ, แตงซูกินี, สายพันธุ์ทนทานต่อการเกิดโรคจากไวรัส

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Abstract

To determine whether a highly-virus-tolerant zucchini (quine) exhibits an efficient reactive oxygen species scavenging system under drought stress. Drought responses of quine were investigated after stress and recovery periods. The drought condition was performed by withholding water for 10 days during late vegetative development, and well-watered condition was used for the control. Glutathione and cysteine accumulation in young leaves were detected on day 10 after stress and day 14 after re-watering. Drought stress significantly induced the increase of total glutathione concentration on stress leaves by 153.29%, while quine could maintain total cysteine and the oxidized cysteine contents at the non stressed control. After seven days of recovery from stress, five weeks-old quine would exhibit various visual characteristics such as undeveloped shoot, short internodes, short petioles bearing non-expanded leaves and chlorosis leaves. In addition, six weeks-old quine produced the lower number of both flowers and developing fruits, compared to control plants.

Further correlation analysis of stressed plants revealed that total cysteine content was significantly highly associated with total glutathione content. Whereas, the negative correlation, r=-0.717, between total glutathione and the oxidized glutathione was observed at the 0.05 level. It is suggested that cysteine accumulation might be contributed for the glutathione production for scavenging purpose under stress condition. It is also confirmed that plant grown under drought stress at vegetative growth exhibited significantly reduced shoot and reproductive organ growth.

Keywords: glutathione, cysteine, drought, zucchini, a virus-tolerant cultivar

1. Introduction

Abiotic and biotic stresses affect both plant vegetative and reproductive developments. Drought stress is a limiting factor for plant growth and crop productivity and may be associated with other conditions such as osmotic, heat and salinity stresses. Consequently, stomatal closure is response of some plants to drought and causes primarily limitation to carbon dioxide uptake in photosynthetic process and expose chloroplasts to excess excitation energy, especially hight light (Smirnoff, 1993). Environmental stresses promote the productions of reactive oxygen species (ROS) such as superoxide, singlet oxygen, hydrogen peroxide and hydroxyl radical (Allen, 1995; Bian & Jiang, 2009).

These ROS lead to lipid peroxidation of membrane bound organelles and cells, and can damage proteins, enzymes, nucleic acids and carbohydrates (Zhang & Kirkham, 1996; Foyer & Noctor, 2011). It was proposed that plants might use four different mechanisms to cope with drought stress including drought escape, drought tolerance, drought recovery and drought avoidance (revied by Gowda et al., 2011).

Plant develops enzymatic [e.g. catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (AP), glutathione reductase (GR)] and non-enzymatic (e.g. reduced ascorbate, reduced glutathione) defense mechanisms protecting oxidative damage of cells. In enzymatic system, SOD catalyzes the dismutation of superoxide anion to hydrogen peroxide, then CAT can eliminate hydrogen peroxide from cells. In non-enzymatic part, ascorbate and glutathione are directly involved through the ascorbate-glutathione cycle of the scavenging system with the presence of some important enzymes such as AP and GR (Smirnoff, 1993; Allen, 1995; Bian & Jiang, 2009). Glutathione (GSH) is an important non protein thiol antioxidant which detoxifies ROS and hydrogenperoxide (Noctor et al., 2011). It can be synthesized from three amino acids including cysteine, glutamate, and glycine (Zechmann & Mueller, 2008). In zucchini or courgette plant (*Cucurbita pepo* L.) which is an economically important crop, most glutathione is generally maintained in its reduced form (GSH) and is consistent with most other plant tissues. GSH plays an important role in reacting with the hydroxyl radical to prevent the oxidation of essential thiol groups (Wang, 1995).

Reports have been undertaken to study effects of low temperature on zucchini during vegetative and reproductive developments, as well as the fruit storage. For example, Tartoura & Yousef (2011) concluded that squash (*C. pepo* L.) exposed to low temperature stress and organic compost treated condition exhibited high level of total ascorbate and glutathione. It was associated with low temperature in stress squash. For zucchini squash fruit (*C. pepo* L., cv., Giambo). It was found that it can activate ROS regulatory systems such as a decrease in hydrogen peroxide, an increase in the activity of CAT and peroxidase, in early stage at 10°C storage to prevent the occurrence of early visible damage in the peel (Gualanduzzi et al., 2009). In addition, studies showed that glutathione level was increased when subjected to drought stress for example sunflower (Sgherri & Navari-Izzo, 1995) and cowpea (Cruz de Carvalho et al., 2010).

Zucchini can be naturally infected by zucchini yellow mosaic virus (ZYMV), destructive virus for cucurbit crop, which can affect its production. It was demonstrated by Zechmann & Mueller (2008) that a highly-virus-tolerant cultivar of zucchini termed quine, infected with ZYMV exhibited the elevated glutathione levels in almost cell compartments of leaves and roots. It was also suggested that the elevated levels of glutathione might be involved for the tolerance during ZYMV infection.

Information obtained from drought responses and non-enzymatic antioxidants system of highly-virus-tolerant zucchini may be limited. As drought stress and virus infection impacts plant growth and productivity, understanding the response under these conditions is important to obtain some valuable evidences for its adaptation. The purpose of the present study was to determine whether a highly-virus-tolerant cultivar exhibits an efficient ROS scavenging system of non-enzymatic defense mechanisms during water deficit. Glutathione and its precursor, cysteine, accumulation in leaves of zucchini in response to drought and recovery periods were determined.

2. Materials and methods

2.1 Plant material

Seeds of a highly-virus-tolerant cultivar (‘Quine’) of zucchini (*C. pepo* L.) were received from Syngenta (Syngenta Seeds SA, Madrid, Spain). Seeds were germinated on a humid perlite cloth and the seedlings were grown in single pots filled with vermiculite. They were maintained in the growth room under a photoperiod of 12 h (PAR 400-700 nm) with a light intensity between 130 and 170 $\mu mol.m^{-2}.s^{-1}$ and 22°C/18°C day/night) temperature. The experiment was conducted at Institute of Plant Sciences, University of Graz, Austria.

2.2 Experiment

The experiment consisted of a randomized complete design with two treatments. The plants were grown for a total of 32 pots. All seedlings were irrigated daily for 16 days, and the plants were further divided into two groups. Well-watered plants were used for control condition. For drought stress treatment, irrigation was with held for 10 days, and the drought-stressed plants were re-watered for 14 days. Eight replications of the youngest fully expanded leaves were collected after 10 d of stress (10 D) and 14 d re-watering post drought (10 D/14 R) for glutathione and cysteine determination.

2.3 HPLC quantification of total contents of thiols

Extraction and determination of total and oxidized thiols were carried out on leaves from control, 10D and 10D/14R plants as described by Kranner & Grill (1993). About 50 mg of lyophilized materials was extracted with 3 ml of 0.1 M HCl (cooled) prepared with 0.08 g polyvinylpyrrolidone (PVPP) for at least 12 hours. Thiols were separated on Grom®Spherisorb ODS-2 250x4.6 mm 50 µm column, and detected with a fluorescence detector (excitation : 380 nm, emission : 480 nm). Solvent A consisted of 0.25% (v/v) acetic acid, pH 3.9 and solvent B was HPLC-grade methanol.

The concentration of solvent B was increased from 10% to 18% in 20.5 min at a constant flow rate of 1 $mLmin^{-1}$, then to 100% at 21 min. After 35 min the initial solvent B concentration was set to re-equilibrate the column prior to the next injection. The concentration of low molecular weight thiols and disulphides in leaf extracts were calculated using calibration curves, and expressed as nmol per g dry weight.

2.4 Statistical analysis

Statistical analyses (Duncan Multiple Range Test and correlation coefficients) were conducted with the program SPSS. Differences at the 0.05 level were considered significant.

3. Results and Discussions

Drought responses in the highly-virus-tolerant cultivar of zucchini, termed quine, were investigated. The irrigation with held for 10 days during vegetative growth was prepared for drought treatment. Vermiculite sampling was done on day 10 after stress for moisture content analysis. It was found that the moisture decreased from about 44% of the normal condition to about 13% under stress, which was approximately 70% less than the control as shown in Figure 1. These data indicated that quine was grown in the severe drought under this study, and the treatment caused the plant to be completely wilted within 10 days of exposure (Figure 2A. & 2B.).

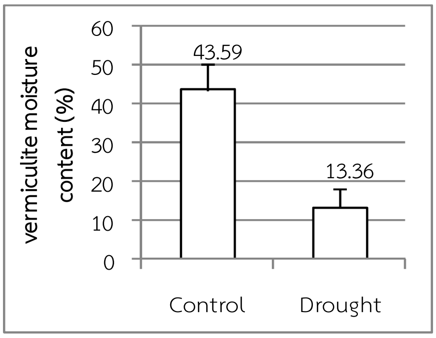

Figure 1 Vermiculite moisture content (%) of control condition and after 10 days stress.

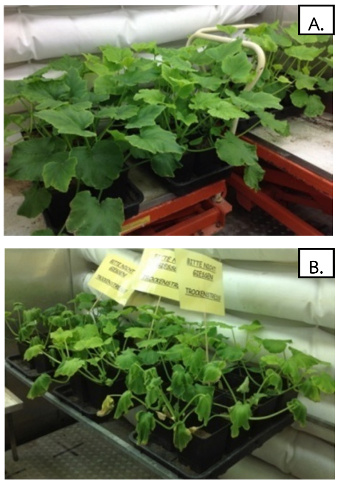

Figure 2 Photographed of quine plants grown under control condition (A.) and exposed to drought for 10 days (B.)

Glutathione and its precursor, cysteine accumulation were detected on day 10 after stress and day 14 after re-watering, and the percentage decrease or increase in the concentration was analysed as shown in Table 1. In case of the precursor, it showed no significant effect on total cysteine content (Figure 3A.) and the percentage of oxidation of cysteine (Figure 3B.) by drought stress in both stress and non-stress treatments. Drought stress significantly induced the increase of total glutathione accumulation on stress leaves by 153.29%, and after re-watering it was minimized to the non-stress control's level (Figure 3C. & Table 1). Elevated glutathione concentration may contribute to drought tolerance by increasing the capacity of plant's protection mechanisms. Typically, glutathione is synthesized in two ATP dependent steps, and the precursor including cysteine, glutamate and glycine are used in the process which are directly involved through the ascorbate-glutathione cycle with the presence of enzymes AP and GR (Zechmann & Mueller, 2008).In poplars (*Populus euramericana*) exposed to artificial drought condition by 100 mmol.L^{-1} mannitol, drought-tolerant clone exhibited a more efficient ROS scavenging system than drought-sensitive clone. AP could be the starting enzyme which reduces H_2O_2 to water of ascorbate-glutathione cycle, it was enhanced in drought-tolerant clone grown under mannitol treatment, while GR was not affcted.The increasing of total glutathione concentration may be earlier response in short term response (Edjolo, 2001). However, detailed analysis on gene expression by Bian & Jiang (2009) revealed that the expressions of glutathione reductase gene (*GR*) was up-regulated in response to drought and remained that levels after recovery on leaves of Kentucky bluegrass.

Glutathione is the most abundant low molecular weight thiol found in most eukaryotic organisms (Maughan & Foyer, 2006). It plays an important role on the cellular pool of non-enzymatic metabolites (Tartoura & Yousef, 2011). Our findings indicated that the quine could maintain the level of total cysteine which is required for the first step of glutathione synthesis. In addition, antioxidant activity may relate to stress tolerance by maintaining an ability to minimize oxidative damage on cell compartment, and recovery metabolic processes.

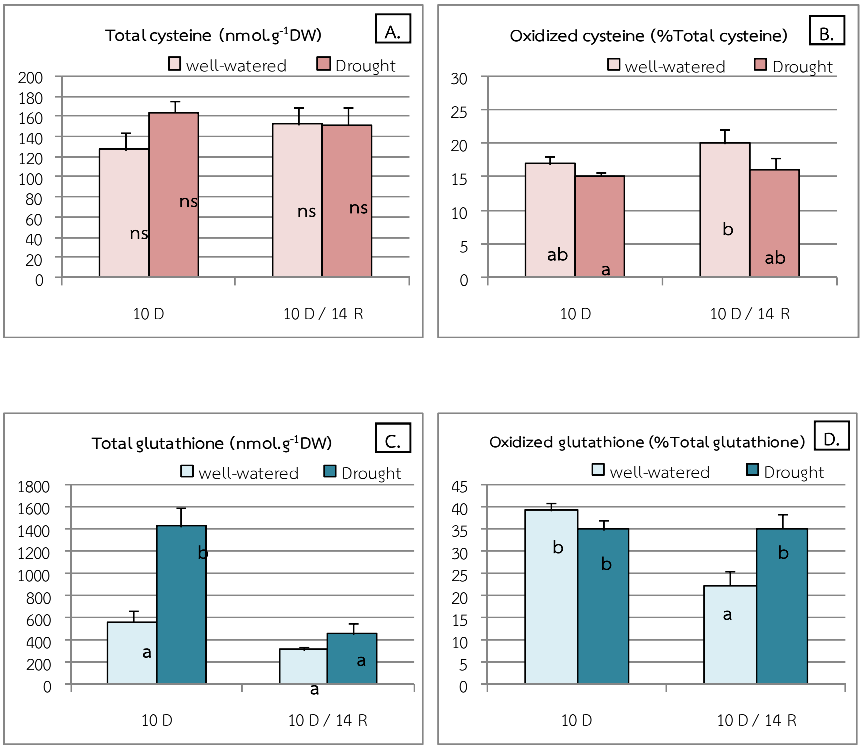

Figure 3 Quantification of total cysteine (A.) and total glutathione (C.) (nmol.g^{-1}DW), and percentage of oxidation of cysteine (B.) and glutathione (D.) (% of total form) of young leaves of 2-weeks-old zucchini after 10 days drought and then 14 days re-watering.

Note: Values are means with standard errors, significant differences (P< 0.05) are indicates by different lowercase letters within the same figures, and ns represents not significant.

Table 1

Percentage decrease or increase in the concentration of stress plants in comparison with the control plants according to values in Fig. 3.

Metabolites	% decrease / increase in concentration		Metabolites	decrease / increase in percentage oxidation	
	day 10 of drought	day 14 of re-watering		day 10 of drought	day 14 of re-watering
Total cysteine	28.53	-0.38	oxidized cysteine	-1.75	-3.87
Total glutathione	153.29*	44.90	oxidized glutathione	-4.28	12.94*

* Difference is significant at the 0.05 level

The percentage of oxidation after stress peroid, expressed as a percentage of total glutathione was 39.18% in control plants, which was not significantly different from stress plants.It meant that the proportion of total glutahione and the oxidized formed was kept constant during drought. The increase of total glutathione level was consistent with the percentage of oxidation on stress leaves, and could maintain the cellular redox status in responses to drought stress. Report has been proposed that the oxidized forms of glutathione (GSSG) and non-glutathione thiol (RSH) contents were low in treated zucchini squash at 15°C for 2 days prior to storage at 5°C and changed only slightly throughout the storage. Consequently, a higher ratio of GSH to GSSG was found in the preconditioned squash than in the control. It suggested that the effectiveness of temperature preconditioning resulting on the percentage of oxidation may reduce chilling injury of fruit during postharvesting storage (Wang, 1995). Research in drought-tolerant cowpea cultivar also reported that cellular redox status capacity depends on the cultivar and related to cellular viability of plants under stress (Cruz de Carvalho et al., 2010).

Information on Figure 3D. and Table 1 showed that oxidized form of glutathione was enhanced in the drought stress leaves by approximately 12.94% oxidation which may be due to the increasing of ROS in response tore-watering compared to the control. It was reported in Kentucky bluegrass that high level of leaf superoxide production and root hydrogen peroxide were detected after recoveryfrom stress (Bian & Jiang, 2009). For drought tolerance improving, recent work by Wu et al. (2014), revealed that Mo application under polyethylene glycol stimulated drought stress improved the water utilization capacity in wheat. This condition enhanced photosynthetic rate, dry matter, grain yield and water use efficiency, and decreased transpiration rate and water loss of wheat under drought stress. Additionally, the contents of nonenzymatic antioxidants content such as ascorbic acid, reduced glutathione and carotenoid were significantly increased.

After rewatering, the stressed plant showed the potential on recovery ability during reproductive stage. Flower and fruit developments were then apparent in these plants. Results showed that a week recovery from stress, 5 weeks-old quine would exhibit various visual characteristics such as undeveloped shoot, short internodes, short petioles bearing non-expanded leaves and chlorosis leaves, while number of leaves was comparable between the two treatments (Figure 4A. &4B.). It was observed that 6 weeks-old quine produced the lower number of both flowers and developing fruits, compared to the non stress control (Figure 4C.; 5A. & 5B.).

These findings suggest that possibility of recovery of the stressed quine can be observed after rewatering. It seems that plant adaptation occurred during early stage of recovery from stress. It would suggest that repsonses due to stress depends on various factors such as plant species or cultivars, timing of stress, stress duration and intensity of stress.

Further correlation analysis of stressed plants (Table 2) showed that total cysteine content was highly associated with total glutathione content (r=0.937**) and the oxidized glutathione content (r=-0.781*). The negative correlation, which was -0.717 and significant at the 0.05 level, between total glutathione and the oxidized glutathione was evident in stress plants. In case of drought tolerant species for example *Oudneya africana*, the Sahara desert plants, the analysis of principal components in antioxidant defence mechanism have been examined. A positive and significant relationship between the redox status (GSH/GSSG; ascorbic acid (ASC)/ dehydroascorbic acid (DHA)), GR (EC 1.6.4.2), the malate dehydrogenase (MDH; EC 1.1.1.37) and NADP-depend isocitrate dehydrogenase (NADP-ICDH; EC 1.1.1.42) under long term treatment were observed. This was stated that the ASC–GSH cycle efficiency was mainly responsible with extreme dry conditions. And, longer exposure to medium stress has a strongereffect than shorter periods at higher intensity (Talbi et al., 2015)

Table 2

Correlations between the concentrations (nmol.g^{-1}DW) of total cysteine, total glutathione, oxidized cysteine and oxidized glutathione of drought-stressed- and control plants.

Control-plants	cysteine	glutathione	oxidized cysteine	oxidized glutathione
cysteine	1	-.346	.754*	-.580
glutathione		1	-.084	.527
oxidized cysteine			1	-.591
oxidized glutathione				1
Stressed-plants	cysteine	glutathione	oxidized cysteine	oxidized glutathione
cysteine	1	.937**	.539	-.781*
glutathione		1	.382	-.717*
oxidized cysteine			1	-.192
oxidized glutathione				1

**. Correlation is significant at the 0.01 level, *. Correlation is significant at the 0.05 level

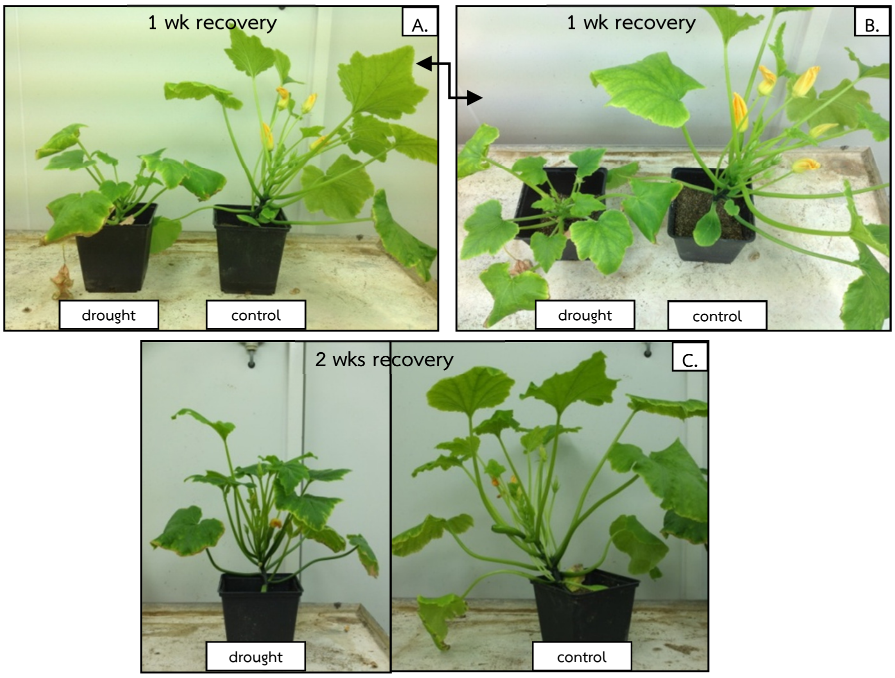

Figure 4 Plants with well-irrigated condition compared to 10 days drought stress, and then recovery for a week of 5 weeks-old plants (A. side view & B. top view) and 2 weeks recovery of 6 weeks-old plants (C.).

Figure 5 Developing fruits of 6 weeks-old zucchini harvested from all replicates of 10 days drought stress followed by 14 days recovery treatments (A.) compared to the well-irrigated plants (B.). Scale bar = 5 cm.

4. Conclusions

For characterising the physiological properties linked to drought stress responses. The highly-virus-tolerant zucchini, quine, showed an ability to cope with drought stress. This study suggested that total cysteine accumulation could be contributed for the glutathione synthesis for scavenging purpose under stress condition. High level of total glutathione could maintain and be responsible for the cellular redox status in responses to drought stress. Based on this study, it was shown that the availabity of glutathione within plants seems to be essential for a successful protection of plants under stress condition.

Furthermore, the quine had potentially adapted after recovery from stress. It is also confirmed that plant grown under drought condition during vegetative growth exhibited significantly reduced shoot and reproductive organ growth.

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