Determination of Superoxide Anion Radical and Antioxidant Enzymes Activities in Viral Infected Vegetable Plants

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Different plants (*Vicia faba* L., *Pisum sativum* L., *Solanum lycopersicum* L., *Cicer arietinum* L., *Lens culinaris* L.) with potential virus symptoms were collected from fields located in the main vegetable production provinces of Azerbaijan. Superoxide anion radicals as well as an activity of antioxidant enzymes (CAT, APO, GR, and SOD) were determined in the study.

Key words: *virus diseases, reactive oxygen species, superoxide anion, antioxidant enzymes, vegetable plants*

INTRODUCTION

Plants must continuously defend themselves against changing and often harmful environmental conditions. One of the factors which affect plants in their environment is biotic stress that results from a battery of potential pathogens such as fungi, bacteria, nematodes, viroids and viruses (Dangl et al., 2001). ssDNA viral diseases are one of the main severe causes for decreased crop productivity in worldwide (Ortiz et al., 2006). Plant viruses with an ssDNA genome have been assigned to two families, *Geminiviridae* and *Nanoviridae* (Stanley et al., 2005; Vetten et al., 2005). Geminiviruses are transmitted by different leafhopper species or the whitefly *Bemisia tabaci* and are assigned, based upon their transmission vector in combination with their respective genome organization, to four genera in the family *Geminiviridae*. Nanoviruses are transmitted by various aphid species in a persistent and non-propagative manner. It causes extensive leaf yellowing, stem and leaf deformation, reduced fruit quality, substantial crop loss and shortening the life-span of vegetable plants. The probable cause of decay of virus infected plants not only the virus activity itself, but also the reduced tolerance to repeated unfavorable environmental situations. Therefore, any little but long lasting defect in the biochemical process might have determinant role in limiting the lifetime of vegetable species. Plants have evolved complex antioxidant systems in order to protect cellular membranes and organelles from the damaging effects of reactive oxygen species (ROS) (Lee et al., 2007). Antioxidant enzymes and metabolites are located in different plant cell compartments to fulfill their protective function. The key enzymes, superoxide dismutases (EC 1.15.1.1; SODs), are a family of metalloenzymes catalyzing the dismutation of O^{2-} to H_2O_2. SODs can be found in chloroplasts, mitochondria, peroxisomes, and in cytoplasm. Catalases (EC 1.11.1.6; CATs), heme proteins that catalyze the removal of H_2O_2, are located in peroxisomes. Enzymes and metabolites of the ascorbate-glutathione cycle (ascorbate peroxidase (APOD), EC 1.11.1.11; glutathione reductase (GR), EC 1.8.1.7 ;) which is important in H_2O_2 scavenging, are located in organelles and cytoplasm (Dangl et al., 2001; Lee et al., 2007). Antioxidant enzymes were often studied at sites of attempted pathogen attack and in connection with immediate responses of invaded cells (Goldbach et al., 2003).

Our study is focused on some stress responses of leaf antioxidant system and the possibility of showing the production of ROS in vegetable leaves infected ssDNA viruses. Here we present summarized results of a work-in-progress.

MATERIALS AND METHODS

In order to detect the presence of ssDNA virus infection during summer and autumn 2009-2011, vegetables with stunting and yellowing symptoms were collected from fields located in the main vegetable production provinces of Azerbaijan (Fig. 1). Virus-free plants were collected from actively growing plants under same field conditions. Collected plant samples presented symptoms of potential virus infection were immediately frozen in liquid N_2 and stored at –20 °C. The extent of viral infection was determined by DAS-ELISA in homogenates of the leaves of infected plants using polyclonal antibodies raised against the respective pathogens and the presence of viral infection was confirmed by two molecular methods (Polymerase chain reaction and Rolling circle amplification).

As known, the level of plant resistance to viral diseases provides many physiological and biochemical parameters responsible for maintaining the viability and alterations in plant metabolism under stress conditions. On this basis, histochemical study of the possible presence of superoxide anion and activity of antioxidant enzymes in virus infected plant leaves were studied in the present work.

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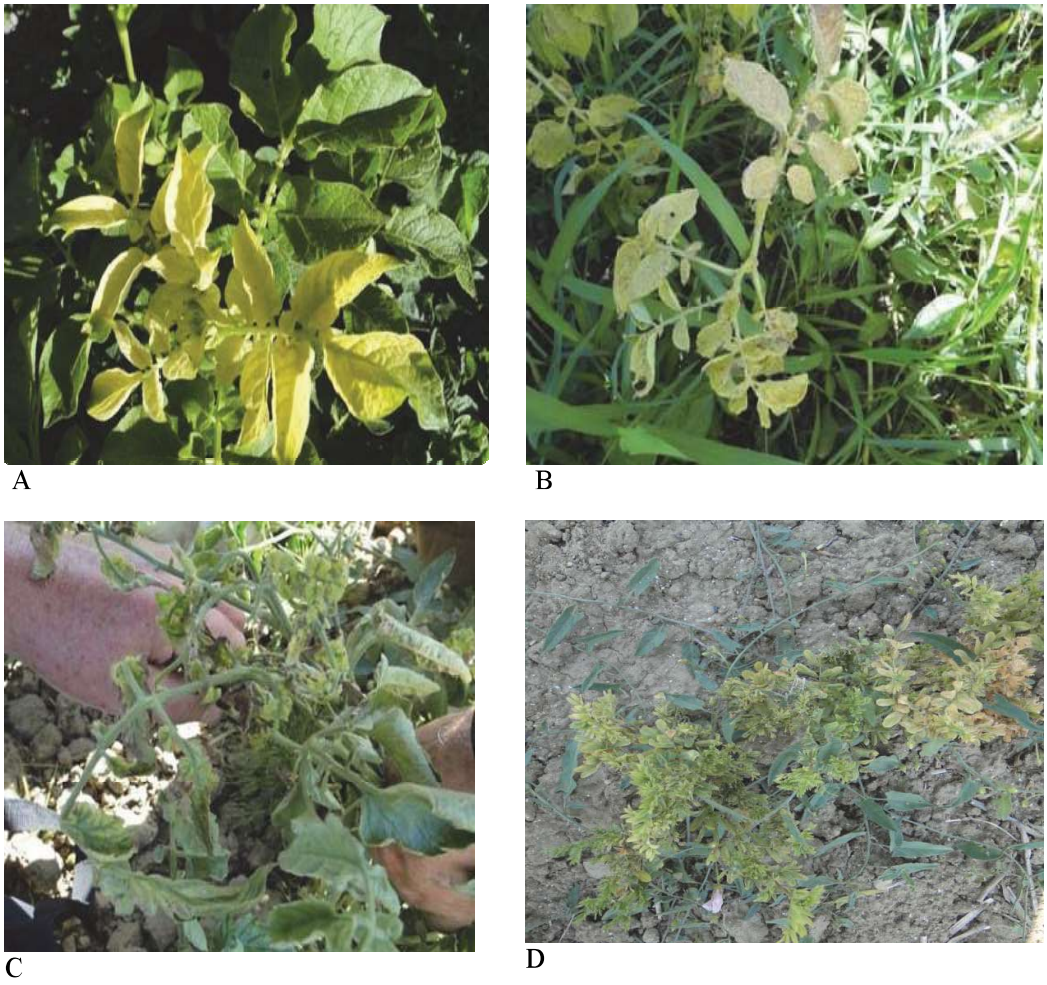

Fig. 1. Symptomatic plants collected from fields associated with virus infestation showing ssDNA virus-like symptoms such as and leaf deformation, stunting and yellowing. A - *Vicia faba* L.; B - *Pisum sativum* L.; C - *Solanum lycopersicum* L.; D - *Lens culinaris* L.

Histochemical staining of superoxide anion radical. Histochemical staining for ROS accumulation was conducted as previously described (Fryer et al., 2003; Kariola et al., 2006; Mahalingam et al., 2005) with some modifications. For superoxide determination, the leaf samples were immersed in 6 mM NBT solution containing 50 mM sodium phosphate (pH 7.5) for 12 h in the dark. To detect ROS reaction were stopped by soaking the leaves with lacto-glycerol-ethanol (1:1:4 by vol) and boiling in water 5 min, and the cleared leaves were preserved in 50% ethanol and photographed.

Enzyme extraction and activity determination. Enzyme extract was prepared by homogenizing fresh leaf material with a pestle in an ice-cold mortar with 0.05 M Na_2HPO_4/NaH_2PO_4 (pH 7.0) buffer. The homogenates were filtered through four layers of cheesecloth and then centrifuged at 4°C. The supernatant were collected and used for the assays of enzymatic activities.

CAT. The activity of catalase was determined as a decrease in absorbance at 240 nm for 1 min following the decomposition of H_2O_2 as described by Kumar and Knowles (Kumar and Knowles, 1993). The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 15 mM H_2O_2 and reaction was initiated by adding enzyme extract.

APO. The activity of ascorbate peroxidase was assayed according to Nakano and Asada (1981). The assay mixture consisted of 0.05 mM ASA, 0.1 mM H_2O_2, 0.1 mM EDTA, 50 mM sodium phosphate buffer (pH 7.6), and 0.3 mL enzyme extract. The activity was measured as a decrease in absorbance at 290 nm for 30 sec.

GR. Glutathione reductase activity was determined at 340 nm for 10 min in 1 ml reaction mix-

ture containing 100 mM potassium phosphate buffer (pH 7,8), 1 mM EDTA, 0,2 mM NADPH and 0,5 mM GSSG (Yannarelli et al., 2007).

SOD. Superoxide dismutase activity was estimated by using SOD Assay Kit-WST (Sigma-Aldrich, USA). The absorbance was recorded at 450 nm and one enzyme unit of SOD activity was defined as the amount of enzime recuired to cause 50% inhibition of the rate of NBT reduction.

RESULTS AND DISCUSSION

ROS generation is a common feature in both incompatible and compatible plant-pathogen interactions. The oxidative burst observed in the initial stages of incompatible interactions (Jabs et al., 2000) is responsible for the induction of defense reactions leading to hypersensitive responses and the development of systemic acquired resistance (SAR) (Wojtaszek et al., 1997).

In this work it was investigated the possible role of reactive oxygen species in plant protection against viral infection by detecting the presence of superoxide anion $O^{2\cdot-}$ in places of infection with the use of the nitroblue tetrazolium (NBT). Accumulation of insoluble blue-colored formazan complex (reduced NBT) is an indicator of generation of ROS, in particular of superoxide anion. This accumulation was observed in infected leaves after infiltration. After this period of time, staining declined rapidly, preceding the apparition of necrosis (Fig. 2). Histochemical staining for superoxide production in leaves tissues was based on the ability of O_2^- to reduce nitro blue tetrazolium (NBT) and used to detect *in situ* the production of superoxide radicals (Leath and Rowell, 1966). Detached leaflets from plants subjected to the viral diseases above described and their respective controls were immersed in potassium phosphate buffer (pH 7.8) containing 0.1% NBT and 10 mM sodium azide. Leaflets were vacuum infiltrated as described above during 2 min, incubated for 2 h in the dark (without vacuum) and then immersed in 96% (v/v) ethanol to completely eliminate the chlorophyll.

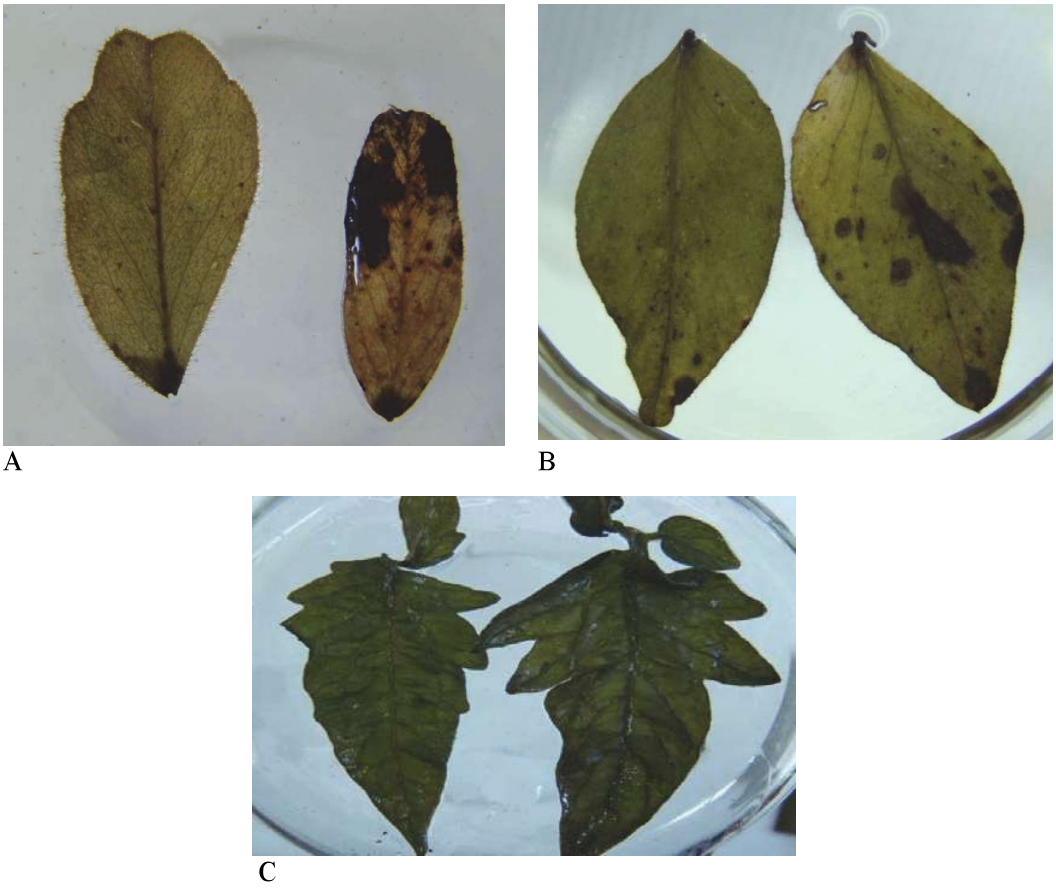

Fig. 2. Histochemical staining of superoxide anion radical in viral infected leaves. A - *Lens culinaris* L., B - *Vicia faba* L., C - *Solanum lycopersicum* L.

Superoxide production was visualized as a purple formazan deposit within leaflet tissues. Leaflets of healthy plants were also infiltrated with 50 mM potassium phosphate buffer (pH 7.8) containing only 10 mM sodium azide and used as control. Superoxide was visualized as a purple discoloration of NBT. Discoloration of leaf was quantified using a digital imaging system (Fig. 3).

We also studied the activities of antioxidant enzymes catalase (CAT), ascorbat peroxidase (APX), superoxide dismutase (SOD) and glutathione reductase (GR) in viral infected plant leaves. These enzymes are known to be involved in an immediate plant defense response. Samples for activity measurements of the antioxidant enzymes were collected during the early stage of the infection, and when the first visible symptoms of the virus infection appeared on the leaves (in early June). As shown in Fig. 2, the activity of antioxidant enzymes in infected leaves generally, was higher than that of comparable healthy leaves.

Analysis of CAT activity in infected leaves showed that this enzyme in all the samples studied had a significant difference compared with the control. CAT activity was 1.4-fold higher (up to 41%) in infected leaves of *Solanum lycopersicum* and 1.27-fold higher (up to 32%) in infected leaves of *Vicia faba* compared to the healthy plants. The most significant differences between the values of CAT activity were observed in infected *Cicer arietinum* samples, where the activity was 2.6-fold higher (up to 163%) compared to the control plants. In infected *Lens culinaris* and *Pisum sativum* samples CAT activity only slightly compared with the control. Analysis of CAT activity in infected *Lens culinaris* leaves showed that this enzyme activity was not significantly different (only up to 17%) compared to the control and accounting 0.42 mmol/mg min respectively. As shown in Fig. 2-panel A, CAT activity in infected *Pisum sativum.* leaves did not seem to be significant affected by viral stress.

APO activity was 1.2-fold higher in infected leaves of *Solanum lycopersicum* compared to the healthy plants and accounting 0.44 mmol/mg min. As shown in Fig. 2, panel B, APX activity in infected leaves of *Vicia faba* and *Cicer arietinum* was slightly increased (up to 24% and 16% respectively), where the activity of CAT had a significant difference compared to the control. The most significant differences between the activities of APX and CAT were observed in infected *Lens culinaris* and *Pisum sativum* samples, where the APX activity was 2.2 and 1.5-fold higher compared to the control plants. This may indicate that the existing functional relationship and competition between the studied enzymes under this viral diseases. Other works also suggest that, along with the activation of SOD and APO in the leaves, observed a sharp decrease in activity of CAT, which may be due to inhibition of the enzyme substrate - H_2O_2. In this case, the high activity of antioxidant enzymes can probably be one of the markers of resistance to the pathogen.

The effects of a viral infection caused an increase GR activity about 1.86-fold higher (up to 72%) and accounting 0.28 mmol/mg min in infected leaves of *Solanum lycopersicum* compared to the healthy plants. GR activity in infected samples *Vicia faba* increased up to 17% compared to the control. In contrast to these options, the GR activity in infected samples of *Cicer arietinum* and *Lens culinaris* did not significantly differ from the control plants. As shown in Fig. 2, panel C, the most significant difference between the GR activities were observed in infected *Pisum sativum* samples, where activity increased approximately 2-fold higher, i.e. up to 96% compared to the control plants.

As shown in Fig. 3, panel D, SOD activity in infected plant leaves had contrast to these options, the actives of Cu/Zn-SOD decreases (up to 46% and 22%) in infected samples *Cicer arietinum* and *Lens culinaris* compared to the healthy plants. The most interesting value of SOD activity observed in infected *Pisum sativum* samples, where the activity did not differ from the control and accounting 0.22 unit/mg proteins.

Plant-virus interaction may result in a host hypersensitive response or in systemic symptoms (Hammond-Kosack and Jones, 2000; Hernandez et al., 2001). One of the earliest responses of plant cells to pathogens is the production of reactive oxygen species (ROS). The typical ROS detected are superoxide radicals ($O^{2\bullet}$) and hydrogen peroxide (H_2O_2) (Johansen et al., 2001; Kombrink and Schmelzer, 2001). ROS play a crucial role during pathogenesis. They are involved in the hypersensitive response typical for plant-pathogen incompatible interactions. They can limit the spread of pathogen by strengthening plant cell walls and/or by killing pathogens directly (Sahoo et al., 2007; Salazar et al., 2006). However, ROS act as cytotoxic compounds, too. Plants have evolved complex antioxidant systems in order to protect cellular membranes and organelles from the damaging effects of ROS (Paranidharan et al., 2003; Subr et al., 2006).

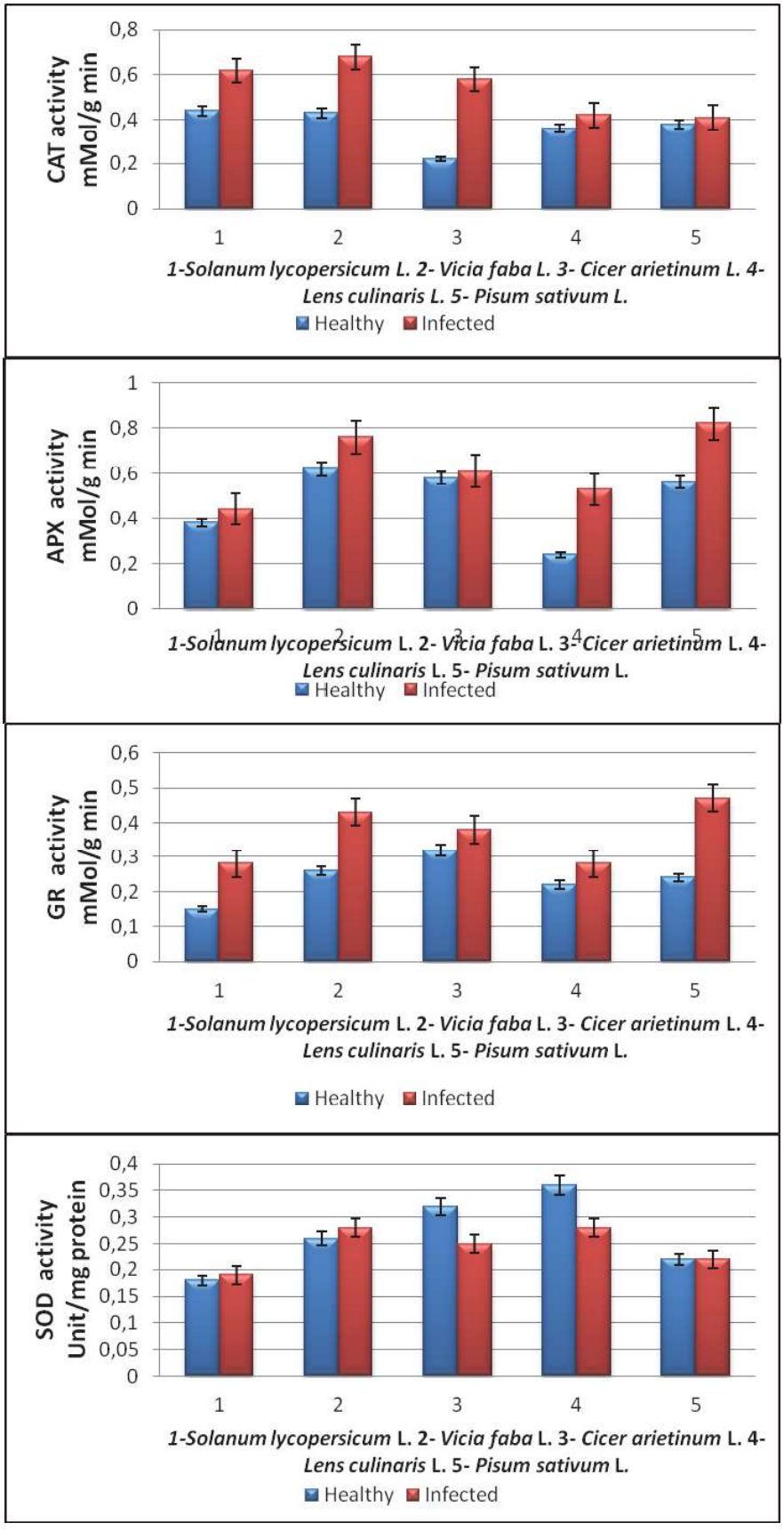

Fig. 3. Activities of catalase, ascorbat peroxidase, glutathione reductase and superoxide dismutase in viral infected plant leaves.

These results can suggest possible targets for the enhancement of stress tolerance in crops by genetic engineering. The data presented here might be used for monitoring biotic stresses in field grown plants and help to selecting resistant varieties for viral diseases.

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Viruslarla Yoluxmuş Tərəvəz Bitkilərində Superoksid Anion Radikalların və Antioksidant Fermentlərin Aktivliklərinin Təyini

Tədqiqat obyekti kimi *Vicia faba* L., *Pisum sativum* L., *Solanum lycopersicum* L., *Cicer arietinum* L., *Lens culinaris* L. bitkiləri seçilmişdir. Potensial virus simptomlarına malik olan bitkilərdə superoksid anionu radikalları və antioksidant fermentlərin (KAT, APO, GR, SOD) aktivlikləri tədqiq olunmuşdur.

И.М. Гусейнова, Н.Ф. Султанова, Д.А. Алиев

Определение Супероксидного Анион Радикала и Активность Антиоксидантных Ферментов в Зараженных Вирусами Овощных Растениях

В качестве объекта исследования были выбраны растения *Vicia faba* L., *Pisum sativum* L., *Solanum lycopersicum* L., *Cicer arietinum* L., *Lens culinaris* L. с потенциальными симптомами вирусных болезней. Супероксидный анионный радикал, а также активность антиоксидантных ферментов (КАТ, АПО, ГР, СОД) были исследованы в данной работе.