

PARTIAL PURIFICATION AND CHARACTERIZATION OF NEWLY EXPRESSED GUAIACOL PEROXIDASE FROM DEHYDRATED SEEDLINGS OF HORSE GRAM (*Macrotyloma Uniflorum*) TISSUE CULTURE

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ABSTRACT

Accumulation of reactive oxygen species (ROS) is a predominant behavior in the most plants when subjected to any abiotic stresses. Seedlings of horse gram tissue culture stressed by dehydration responded by expression of a new isoform of guaiacol peroxidase (GPOX) comparison with unstressed seedlings. This new GPOX was purified by passing the ammonium sulfate fraction of extract through 7-12 % gradient resolving gel of PAGE at 4 °C. A small part of the gel was cut and subjected to activity staining of peroxidase. By aligning the stained and unstained gels, the portion of unstained gel corresponding to the enzyme band have been excised. The enzyme was recovered by electro-eluting technique using Tris buffer, pH 6.8 for 45 min, and partially characterized. The purified GPOX has approximately 43 kD molecular mass; it was stable at low pH and it was maximally active at pH 6. The enzyme was more sensitive to high temperature, and its optimum temperature was at room temperature. Kinetic constants were determined, K_m values was 15 mM and 0.6 mM of for guaiacol and H_2O_2 respectively while V_{max} was 582.9 U/mg for guaiacol and 122.5 U/mg for H_2O_2 . The K_m values indicate that the enzyme is highly specific to hydrogen peroxide and showed higher substrate affinity than previously reported GPOXs. This fact suggest that the new isoform of GPOX which expressed during dehydration stress is as part of the defense system in the plant which may enhance scavenging of ROS and assist the stressed plant to keep survive.

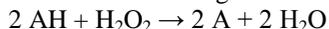
Key words: Dehydration; Tissue Culture; Antioxidant Enzymes; Guaiacol Peroxidase; Horse Gram.

Abbreviations: H_2O_2 : Hydrogen Peroxide, PEG: Polyethylene Glycol; ROS: Reactive Oxygen Species.

INTRODUCTION

Peroxidases (EC 1.11.1.7) are a group of enzymes widely distributed in the plant kingdom (Van Huystee, *et al.*, 1987) and can be found in vacuoles, tonoplast, plasmalemma and inside and outside the cell wall. These enzymes have a variety of functions as evidenced by the presence of several isoenzymes in plant cell compartments (Vitali Alberto *et al.*, 1998).

They are involved in plant hormone regulation (Gaspar T. 1986), defense mechanisms (Hammerchmidt R. et al., 1982; Kolattukudy P. E. et al., 1992; Bradley D. J. et. al., 1992), control of cell elongation, polymerization of extension (Ahmed N. et. al., 1995), cross-linkage of cell wall polysaccharides (Fry S C. 1986), lignin biosynthesis (Gross G G 1980) and the suberization processes (Gaspar T. et al., 1991). Peroxidase enzymes catalyze oxidation-reduction reactions, such as the following:



They reduce H_2O_2 to water while oxidizing a variety of proton donor compounds (AH) such as diphenols, polyphenols aminophenols, among others. Peroxidase has a variety of substrates which use H_2O_2 as electron acceptor for catalyzing different oxidative reactions under various environmental stresses. Peroxidases which oxidize guaiacol (*o*-methoxyphenol), as a commonly used reducing substrate *in vitro*, are referred to as guaiacol peroxidases (GPOX). Peroxidase has been characterized from several plants including horseradish (*Armoracia rusticana*), peach (*Prunus persica*), yam (*Alocasia macrorrhiza*), sweet potato (*Ipomoea batatas* ex L., Lam.), turnip (*Brassica campestris rapifera*), zucchini (*Cucurbita pepo*) and others (Ounigpipat et. al., 1995). For vegetables and fruits, various types of isoperoxidases, including the anionic and cationic types with low and high isoelectric point values have been identified as both soluble and membrane bound isoforms (Adams, 1997).

In this paper, we describe the partial purification and properties of the newly expressed guaiacol peroxidase under dehydration stress and some kinetic characteristics, as well as sensitivity to thermo stability and pH stability.

MATERIAL AND METHODS

Plant Material and Stress Treatments

Seeds of horse gram, *Macrotyloma uniflorum* (PHG-9 cultivar) brought from University of Agriculture Science GKVK campus Bangalore and sown in vermiculite and used for regeneration tissue culture. Explants of shoot tips were aseptically inoculated on Murashige and Skoog's (1962) media with L2 vitamins and leguminous media (Phillips and Collins, 1979) on solid agar. After the development of sufficient growth, the calli were subcultured into media containing 10% PEG as a dehydration stress inducer. Well-developed regenerates of 30-35 day old were used for extraction of enzymes and antioxidants. The regenerates grown in the normal media without PEG served as controls.

Crude Enzyme Extraction

Frozen shoots were homogenized with 50 mM sodium phosphate buffer (pH 7.5) containing 1 mM PMSF (Phenyl methylsulphonyl fluoride) and 5 mM β -mercaptoethanol. The homogenate was centrifuged at 10000 rpm for 20 min. The supernatant was used as source of enzymes, antioxidants, and other components. All the steps in the preparation of the enzyme extract were carried out between 0 - 4 °C.

Purification by Preparative PAGE

Crude extract of the enzyme from the stressed tissue culture plants was treated by precipitation by ammonium sulfate (0-60) saturation and subjected to gradient PAGE on a 7-12 % gradient of resolving gel and 5% stacking gel at 4 °C. A portion of the gel was cut and subjected to activity staining for peroxidase. After confirming position of the peroxidase band by aligning the stained gel with unstained gel, the gel corresponding to the

enzyme was excised. The gel was further cut into smaller pieces and electro-eluted into 20 mM Tris (hydroxymethyl aminomethane) buffer, pH 6.8 for 45 min. The eluted buffer was used for determination of protein content and the activity of peroxidase.

Enzyme Assay

Peroxidase was assayed by determining the rate of guaiacol oxidation in the presence of H_2O_2 at 470 nm (Rao *et al.*, 1996). ($\epsilon_{470} = 26.6 \text{ mM}^{-1}\text{cm}^{-1}$). One unit of peroxidase is defined as the amount of enzyme that caused the formation of 1 mM of tetra-guaiacol per minute. POX isozymes separated on 9% native acrylamide gels were incubated in a mixture of *o*-dianisidine-HCl in acetate buffer (pH 5.5) for 30 min at room temperature. Gels were then transferred to 100 mM H_2O_2 until visible bands developed. Soluble protein content was determined according to the method of Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as the standard. The purified protein was assessed for purity by SDS-PAGE. (Sodium Dodecanyl Sulphate – Polyacrylamide Gel Electrophoresis).

Electrophoretic Analysis

Non-denaturing, discontinuous slab gel electrophoresis was carried out essentially according to the method of Davis (1964), while SDS-PAGE was performed according to Laemmli (1970).

Determination of Peroxidase Molecular Weight

The purified peroxidase was subjected to SDS-PAGE along with standard molecular weight markers (14.4, 18.4, 25, 35, 45, 66.2 and 116 kD - *Fermentas*) according to the method described by (Weber *et al.*, 1972). The Log molecular weight and relative mobility were used to estimate the apparent molecular weight of the proteins.

Determination of Isoelectric Point

Isoelectric focusing (IEF) was performed on a 7.5% polyacrylamide gel containing 2% ampholyte pH 3.5-10 (*BioRad*) using tube gel electrophoresis system. Purified enzyme (0.1 mg) was loaded onto the gel maintained at 4 °C. After IEF, the gels were stained for POX activity as given above. The electro-focused gels were stained for proteins after leaching the ampholytes in 5% TCA.

Effect of Temperature and pH on POX Activity

Activity of the purified POX was determined at room temperature over the pH range of 3.5-9 in 50 mM buffers. To study the effect of temperature, 1.0 ml reaction mixture containing enzyme and substrates in cuvettes were incubated at different temperatures (15-60 °C) for 10 min in 50 mM sodium phosphate buffer (pH 7.0) and enzyme activity was measured by monitoring the increasing absorbance at 470 nm.

Determination of Kinetic Constants (K_m and V_{max})

Michaelis-Menten constant (K_m & V_{max}) for substrates guaiacol and H_2O_2 were determined by incubating 0.1 ml of purified peroxidase with different concentrations of guaiacol and a fixed saturated concentration of H_2O_2 and vice-versa. The K_m and V_{max} values were determined from double reciprocal plots of enzyme activity and substrate.

Statistical Analysis:

All data are expressed as means of triplicate experiments unless mentioned otherwise. Comparisons of means were performed using *Graph Pad Prism* software. Data were subjected to a one-way analysis of variance (ANOVA), and the mean differences were compared ($P < 0.05$).

RESULTS AND DISCUSSION

Dehydration stress increases the formation of reactive oxygen species (ROS) resulting in lipid peroxidation, denaturation of proteins and nucleic acid damage with severe consequences on overall metabolism (Hansen et. al., 2006). The toxic effects of ROS are counteracted by enzymatic as well as non-enzymatic antioxidants including complex nonenzymatic (ascorbate, glutathione, α -tocopherol phenolic compounds) and enzymatic antioxidants like catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), superoxide dismutase (SOD), polyphenol oxidase (PPO), peroxidase (POX) etc. (Jaleel et. al., 2007). Normally, each cellular compartment contains more than one enzymatic activity that detoxifies a particular ROS. Plant's ability to respond to different environmental cues and adaptation is due to altered expression of genes. When the plants are grown in tissue culture media, they switch their metabolic programs and adapt to new conditions. Horse gram shoot explants were used to regenerate the tissue. The combination of MS media and L2 vitamin mix yielded good shoot growth. The plantlets obtained from tissue culture were analyzed for their ability to respond to dehydration stress.

The ROS produced during applied stress is efficiently processed by antioxidant enzymes. Considerable elevation in the level of guaiacol peroxidase (GPOX) activity, in addition to presence of new expressed isoform of this enzyme in stressed tissue culture compared with non-stressed seedlings indicated that greater degree of stress imposed on the plant and presence of an efficient detoxification system in horse gram.

Peroxidase is one of the key enzymes controlling plant differentiation and development. It is known that this enzyme participates in the construction, rigidification and eventual lignification of cell walls, in the biosynthesis of H_2O_2 , in the protection of plant tissues from damage and infection by pathogenic microorganisms, and in wound healing (Farrell et. al., 1989). The new isoforms of the GPOX observed under dehydration stress in tissue culture regenerates was purified to homogeneity as judged by SDS-PAGE and isoelectric focusing.

Electrophoresis under native conditions indicated that the soluble enzyme is more anionic and is newly expressed under dehydration conditions (Fig. 1A). The crude tissue culture regenerate extract was subjected to preparative PAGE under non denaturing conditions and the anionic GPOX was electro-eluted. The electro-eluted horse gram guaiacol peroxidase (GPOX) exhibited a single band when stained for its activity with *o*-dianisidene: hydrogen peroxide (Fig. 1C) as well as in SDA-PAGE stained with coomassie brilliant blue to detect protein bands (Fig. 1B).

Relative Molecular Mass

The purified protein, on subjecting to isoelectric focusing, got focused at pH 5.2. The pI value of the protein is in agreement with its electrophoretic mobility in non-denaturing PAGE in anionic buffer system. The relative molecular weight of the protein (about 43 kD), was determined by SDS-PAGE based on method described by Weber et. al., 1972. using standard molecular weight (Fig. 2) markers between 14-116 kD (*Fermentas*). Comparison

of the molecular weight of the protein indicated that it is distinct from ascorbate peroxidase isozymes and appeared to be more like heme peroxidase (Shigeoka et. al., 2002). Also, it is similar to isoperoxidases reported from strawberry which exhibited MW of 58.1 and 65.5 kD (Civello et. al., 1995). Two isoform of heme peroxidase enzyme, with molecular weight 38 and 45 kD were reported by Vianello et. al., 1997 while Kumar and Malhotra, 2008, reported 81.5 kD for POX from ber fruit (*Zizyphus mauritiana* Lamk).

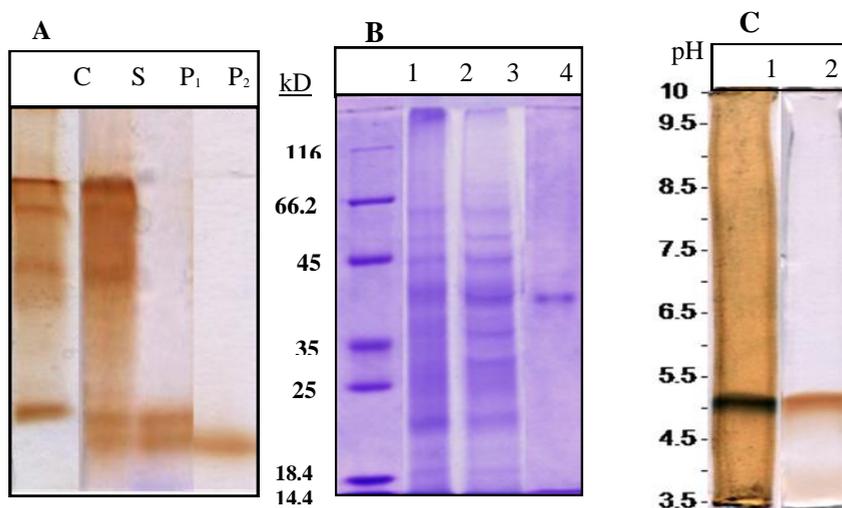


Figure 1: Electrophoresis PAGE Zymograms of (A) Nondenaturation PAGE staining of POX activity in the gel: lane C; Control (non stressed tissue culture seedlings) lane S; dehydration stress seedlings, lane P₁; Partially purified POX by preparative, lane P₂; purified POX by preparative. (B) SDS-PAGE protein staining of lane 1; protein molecular weight markers, lane 2; is crude extract of control tissue culture, lane 3; is crude extract of stress tissue culture, lane 4; is purified new isoform of POX of stress tissue culture and (C) Nondenaturation PAGE of purified POX, 1; IEF protein staining, and 2; is IEF POX activity staining.

Kinetic Analysis

The purified GPOX exhibited a K_m of 15 mM for guaiacol and 0.6 mM for H_2O_2 . (Fig. 3A, 3B, 4A, and 4B) Also V_{max} was 582.9 U/mg for guaiacol and 122.5 U/mg for H_2O_2 . The K_m values indicated that the enzyme is highly specific to hydrogen peroxide. The GPOX exhibited higher substrate affinity than other peroxidases previously reported. A K_m of 20.28 mM and V_{max} of 8,210 U/min was reported for guaiacol, and a K_m of 12.75 mM, and V_{max} of 11,635 U/min for hydrogen peroxide for carambola anionic peroxidase (Maciel et. al., 2007).

K_m values in a millimolar range are typical for peroxidases with artificial substrates like guaiacol. For instance, soluble peroxidases from kiwifruit (*Actinidia deliciosa*) and tomato (*Lycopersicon esculentum*) fruits had K_m values of 7.4 and 10 mM, respectively (Mika and Lüthje, 2004).

Optimum pH and Temperature

Purified GPOX was more stable at lower pH. The enzyme activity was relatively stable in lower and neutral pH nearly constant for 5-6 days at 4 °C. The optimum pH of the enzyme

for guaiacol and hydrogen peroxide was found between pH 5 and 7.0 (Fig. 5 B). From the pH optimum of the enzyme, it is evident that the enzyme is preferentially located in cytosol and not in organelles. However, the enzyme exhibited maximum activity at pH 6.0 at room temperature (Fig. 5B). The purified enzyme was maximally active between 25 to 30 °C (Fig. 5A). It was expressed as a response to dehydration stress, expecting that the enzyme will show thermostability, but it showed the maximum activity at room temperature. This suggests the fact that it cannot participate in cold stress response.

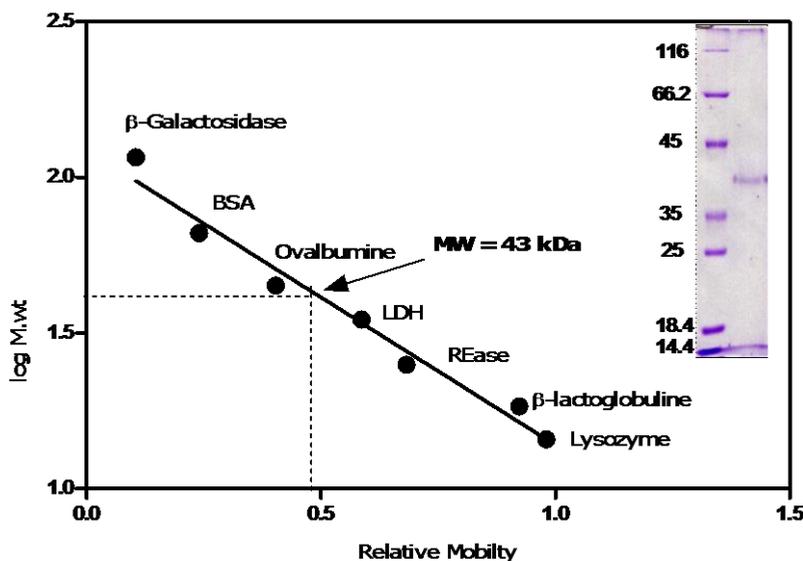


Figure 2: Determination of relative molecular weight of newly expressed POX isoform purified from dehydrated stressed tissue culture seedlings of horse gram by using relative mobility on SDS-PAGE using standard molecular weight markers proteins as presented in the above zymogram.

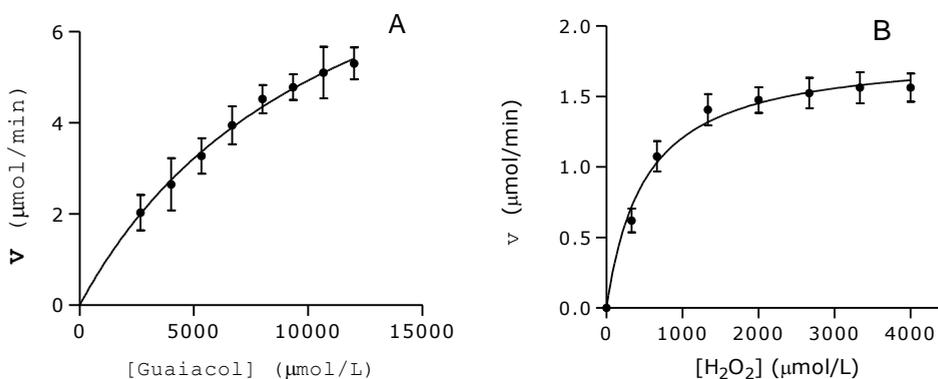


Figure 3: Graphs of Michaelis-Menten (M-M), for purified (HGP) POX using the Hydrogen peroxide- Guaiacol substrate system, (A) M-M plot for POX with varying concentrations of Guaiacol and fixed concentration (0.3 mM) of H_2O_2 , (B) M-M Plot for POX with varying concentrations of H_2O_2 and fixed concentration (0.4 mM) of Guaiacol.

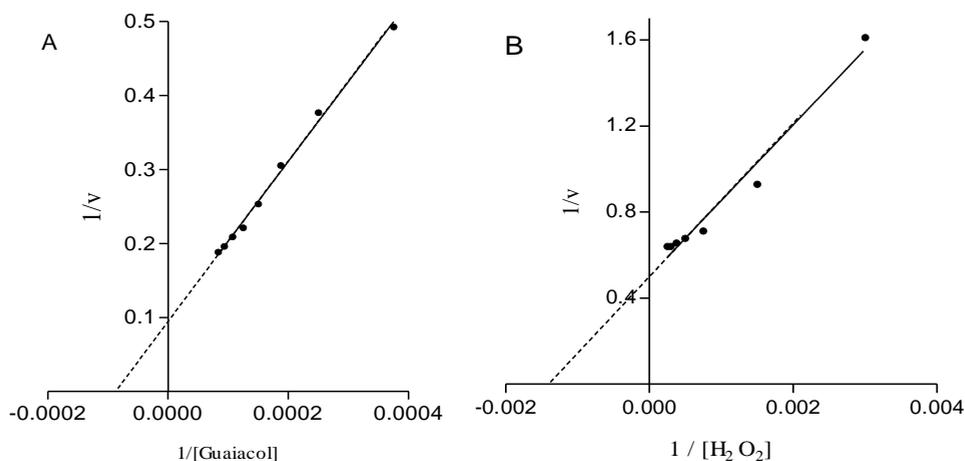


Figure 4: *Lineweaver Burk* (L-B) plots to determine the kinetic parameters of Horse gram newly expressed guaiacol peroxidase (G-POX) (A) Double reciprocal plot for HGP carried out by Assays POX activity with varying concentrations of Guaiacol and fixed concentration (0.3 mM) of H_2O_2 at 25 °C. (B) POX assayed with varying concentrations of H_2O_2 and fixed concentration (0.4 mM) of Guaiacol at 25°C.

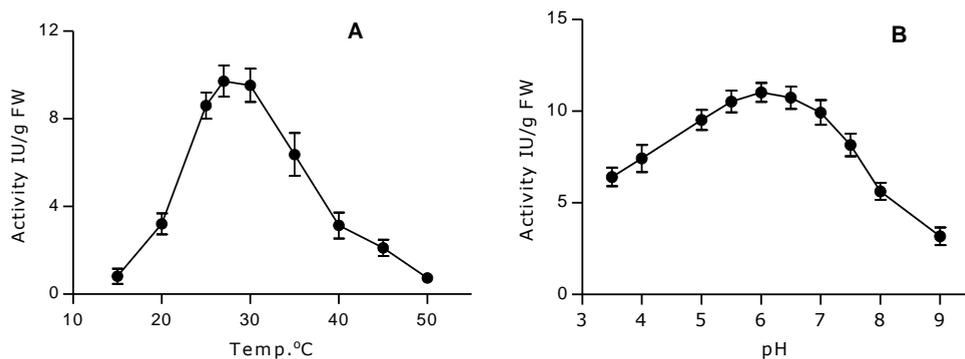


Figure 5: (A) Effect of temperature on purified G-POX. (B); Effect of pH on purified HG-POX activity data presented are average values \pm SE of $n = 2$ experiments.

CONCLUSION

The new isoform of GPOX which expressed in horse gram tissue culture during its subjecting to dehydration stress was purified by preparative method and some of its properties were studied. The obtained results showed the GPOX appearing to be an anionic, and more akin to cytosolic peroxidase, it was more stable at low and neutral pH, but exhibited less stability at higher temperature. also It exhibited higher substrate affinity to hydrogen peroxide and guaiacol, this suggests that the scavenging system of the plant is insufficient to detoxify ROS accumulated during the stress, this made the plant able to express the new GPOX which can participate in the detoxification of ROS, enhance the protection system, and increase the resistance of the plant against dehydration stress.

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تنقية ودراسة جزئية لخصائص أنزيم جواياكول بيروكسيديز المستخلص من نباتات غرام الحصان (*Macrotyloma Uniflorum*) المستنبتة صناعياً أثناء تعرضها لإجهاد الجفاف

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ملخص

يعتبر تراكم مركبات الأوكسجين النشطة (ROS) سلوك سائد في النبات عندما تتعرض لأي من الإجهادات غير الحية. عندما عُرضت نباتات من غرام الحصان مستنبتة صناعياً للجفاف أنتجت شكل جديد من أشكال إنزيم البيروكسيديز المختص بمادة جواياكول بالمقارنة مع النباتات التي لم تخضع للجفاف (المرجع). وقد تم تنقية هذا الإنزيم عبر حقن عينة المستخلص المرسبة بكريتات الامونيوم في هلام الاكريل اميد متدرج التركيز باستخدام هلام تحليل تركيزه ٧ – ١٢ % جزء من الهلام تم قطعه وصباغته لتحديد موقع الإنزيم عليه. ثم تمت مقارنته مع الهلام غير المصبوغ لتحديد الموقع المقابل للإنزيم ثم أخذ الجزء المقابل لموقع الإنزيم وتم إعادة استخراج الإنزيم منه باستخدام تقنية الإزالة الكهربائية. وقد تم دراسة بعض خواص الإنزيم المنقي وكان وزنه الجزيئي حوالي ٤٣ كيلو دالتون، كما أظهر ثباتاً أعلى عند درجات الحموضة المنخفضة وكان أعلى نشاطاً له عند pH = 6 بينما أبدى تأثيراً أكبر عند درجات الحرارة العالية حيث كان أعلى نشاطاً له عند درجة حرارة الغرفة تقريباً.

و كانت قيم K_m حوالي ١٥ و ٠,٦ ملي مول لكل من الجواياكول وفوق أكسيد الهيدروجين على التوالي فيما كانت قيم V_{max} للإنزيم ٥٨٢,٩ وحدة دولية / مجم لمادة الجواياكول و ١٢٢,٥ وحدة دولية / مجم لمادة فوق أكسيد الهيدروجين. وتدل قيم K_m مدى ألفة الإنزيم العالية لمادة فوق أكسيد الهيدروجين حيث وجدت أعلى من ألفة الإنزيمات المدروسة سابقاً. ومن هذه النتائج يمكن الاستدلال بأن هذا الأنزيم المنتج تحت تأثير الجفاف يمثل جزء من نظام الدفاع للنبات و يعمل على تعزيز إزالة مركبات الأوكسجين النشطة ويساعد النبات المجهد على البقاء حياً.