Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

**E-ISSN:** 2278-4136
**P-ISSN:** 2349-8234
JPP 2018; 7(1): 2024-2031
Received: 14-11-2017
Accepted: 15-12-2017

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# Herbicide effects on pigments and antioxidant enzymes of *Portulaca oleracea* L

**S Laxmi, MR Chambhare, S Kadlag, ML Ahire and TD Nikam**

**Abstract**

*Portulaca oleracea* L. (Purslane), is a world's most unscrupulous weed having very strong propagation method, stress tolerance and difficult to control by conventional methods. To ensure its control, we studied the weedy characteristics of *P. oleracea* by analyzing the re–emergence capacity after sunlight exposure for a month and influence of different concentrations of four herbicides *viz*. Oxyfluorfen (Goal), Glyphosate (Roundup), Pendimethalin (Stomp) and 2,4-dichlorophenoxyacetic acid (2, 4–D) on *in vitro* and naturally grown plants. The plants after removing from soil were place without watering and expose to sunlight (maximum intensity 550-600 watt /$m^2$) for a month, showed the rapid re–emergence after plantation in soil and watering. The treatment of 2, 4–D and Oxyfluorfen were drastically reduce the betalain content. Pendimethalin did not have any significant effect on Chlorophyll content and betalain content. Initially, the activity of enzyme superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guiacol peroxidase (GPX) in the herbicide treated plants was higher over the control. Later the sharp decline in enzyme activity was observed with the increase of contact time of herbicide to shoots of *in vitro* and plants grown in natural conditions. Both *in vitro* raised and plants grown in natural conditions were died with the treatment of 5% 2, 4–D and 5% Oxyfluorfen after 4 days of application. Our results provide evidence that *P. oleracea* has strong ability to survive in adverse conditions and 2, 4–D and Oxyfluorfen can be effective herbicide to its control.

**Keywords:** *Portulaca oleracea*, Purslane, betalains, herbicides, antioxidant enzymes

## Introduction

Purslane (*Portulaca oleracea* L.; Portulacaceae) is an annual fleshy weed, most commonly found in waste and barren land. It is well adapted to warm climate, and possess C4 type of photosynthesis [1]. The characteristic weedy nature of *P. oleracea* is attributed due to its ability to produce as many as 10000 seeds which may be dispersed by animals, humans and birds [2, 3]. Naturally, the plant is mainly propagated through seeds but it can also well propagated by the means of fleshy stem fragments. *P. oleracea* has been reported to be salt tolerant as well as drought tolerant which add to their ability to thrive under water deficient and saline conditions [4, 5, 6].

Conventional method used to control weed include manual weed removal, biological control, cultural control and application of chemical herbicide [7]. Chemical herbicide provides relatively easy and effective method to check the population of most of the weeds [8]. Chemical herbicides are of different types and selective in nature depending upon the weed that needs to be controlled. There are two different types of weedicides depending upon applications during the emergence like pre emergence and post emergence herbicides [9]. Pre-emergence herbicides are applied directly to the soil to prevent effective germination of seeds of weeds, whereas post emergence herbicides are applied onto the established weeds to arrest the growth and to kill the weeds [10]. Different herbicides varies in their ability to control the same weed. Chemical herbicides on application are absorbed by the crop plants and weeds; the plants are able to metabolize the herbicides hence they are not affected whereas weeds are not able to metabolize the herbicides which prove to be phytotoxic [11].

Large number of research have been done to understand the physiological and biochemical changes induced in crop plants under the effect of different herbicides; however, meager information is available on the physiological and biochemical responses of weeds under the influence of herbicides application. Therefore in the present study, we have examined the re-emergence capacity of *P. oleracea* after sun drying for a month and effect of different herbicides at varying concentration on pigment content and antioxidant enzyme activities in *in vitro* cultures and field grown plants of *P. oleracea*.

## Material and methods

### Plant material and re–emergence studies

Fresh plants of *P. oleracea* were uprooted from the field of Botanic Garden of the Department of Botany, Savitribai Phule Pune University, Pune and brought to the laboratory. The plants after removing from soil were place without watering and exposed to sunlight (maximum intensity 550-600 watt /$m^2$) for 7, 14, 21 and 28 days. After each treatment the plants were planted in earthen pot (15 cm x 15 cm) containing garden soil. The watering was carried out at every day in the evening and observed for the retrieval of growth.

### Herbicide treatment at field level

The seeds of *P. oleracea* were collected from the plants grown in the field at Botanic garden of the Department of Botany, Savitribai Phule Pune University, Pune (MS) India. The seeds (20 seeds) were sown in earthen pots (15 cm x 15 cm) containing garden soil and watered with tap water as per the requirement. The pots were maintained in the Botanic Garden under natural conditions. After 15 days of germination, 5 uniform grown plantlets were maintained in each pot. After 2 months of growth, the plants were sprayed with different concentrations (1% and 5%) of herbicides (Table 1). For every treatment 5 pots each containing 5 plants was sprayed with respective concentrations of herbicides. The plants sprayed with distilled water were served as control.

### Establishment of *in vitro* cultures and herbicides treatment

The shoot cultures of *P. oleracea* were established using nodal explants obtained from the healthy plants grown in field. The explants were washed thoroughly with 0.01% (v/v) Tween-20 solution followed by sterilized distilled water. Then the explants were surface sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 5 min, and washed 5 times with sterilized distilled water to remove the traces of $HgCl_2$. The surface sterilized nodal explants were cultured onto the Murashige and Skoog [12] medium supplemented with 4.44 µM 6-Benzyladenine (BA) and 30g $l^{-1}$ sucrose. The pH of the medium was adjusted to 5.8 and solidified with 0.8% (w/v) agar-agar (Hi Media, Mumbai, India) prior to autoclaving at 121ºC for 15 min. The shoots cultures were maintained in liquid MS medium containing 4.44 µM BA and used for the experiments. These shoot cultures were subjected to the treatment of different concentrations (0, 1 and 5%) of herbicides (Table 3, 5) separately in liquid MS medium supplemented with 4.44 µM BA. About 4 shoots were inoculated in glass bottles containing 50 ml of MS liquid medium fortified with 4.44 µM BA and respective herbicides concentrations separately. The cultures were maintained under the controlled conditions as described earlier.

**Table 1:** Herbicides common name, chemical name, their family, treatment and mode of action.

| Common name | Chemical name | Family | Treatment (%) | Primary mode of action |
| --- | --- | --- | --- | --- |
| 2,4-D | 2, 4-Dichlorophenoxy acetic acid | Phenoxy acid | 1 and 5% | Auxins type growth regulator |
| Goal | Oxyfluorfen | Diphenyl ether | 1 and 5% | Protox inhibitor |
| Roundup | Glyphosate | Amino acid derivative | 1 and 5% | EPSP synthase inhibitor |
| Stomp | Pendimethalin | Dinitroaniline | 1 and 5% | Microtubule/Spindle apparatus (root growth) inhibitor |

### Determination of chlorophyll and betalain content

Chlorophyll content was estimated by extracting 100 mg of leaf from herbicide treated and control samples in 5 ml 80% (v/v) acetone (Qualigens, Mumbai, India). Then the samples were centrifuged at 5000 rpm for 5 min and the absorbance of supernatant was recorded at 645, 652 and 663 nm on UV-VIS spectrophotometer (Shimadzu - 1601, Japan). Chlorophyll content was calculated as per standard method [13].

For estimation of betalain content, the plant materials (herbicides treated and control samples) were submerged into liquid nitrogen and then ground using mortar and pestle. Finely powdered plant material (500 mg) was mechanically shaken with 10 ml of distilled water for 10 min at room temperature. Then the samples were centrifuged at 10,000 rpm for 15 min. The supernatant obtained was analyzed for betalain content at 538 nm, 476 nm and 600 nm using spectrophotometer. Betalain content was calculated as per the method described by Castellanos–Santiago and Yahida [14].

Betalain contents (mg/g) = $[(A\ (DF)\ (MW)\ Vd/\varepsilon L Wd)]$ where $A$ is the absorption value at the absorption maximum of 535 and 483 nm for betacyanins and betaxanthins, respectively, DF is the dilution factor, $Vd$ is the dried pulp solution volume (mL), $Wd$ is the dried pulp weight (g), and $L$ is the path–length (1 cm) of the cuvette. The molecular weight (MW) and molar extinction coefficient ($\varepsilon$) of betanin [MW 550 g/mol; $\varepsilon$ 60,000 l/(mol cm) in $H_2O$] were applied in order to quantify the betacyanins. Quantitative equivalents of the major betaxanthins (Bx) were determined by applying the mean molar extinction coefficient [($\varepsilon$) 48,000 l/(mol cm) in $H_2O$].

### Antioxidant enzymes assay

#### Extraction

Fresh treated and control samples (500 mg) were homogenized in 5 ml of ice cold 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA (Hi Media, Mumbai, India) and 1% (w/v) Polyvinylpyrrolidone (PVP; Hi Media, Mumbai, India) with chilled mortar and pestle. The homogenate was filtrated with single layered cheese cloth and centrifuged at 10,000 rpm for 20 min at 4ºC. An appropriate aliquot/dilution of the supernatant was used as a crude enzyme(s) for determination of antioxidant enzyme activities. Soluble protein content in the enzyme extract was determined according to Lowry *et al*., [15] using bovine serum albumin (BSA; Merck, Mumbai, India) as a standard.

### Superoxide dismutase (SOD) assay (EC 1.15.1.1)

The total SOD was assayed according to Becana *et al*., [16] by inhibition of the photochemical reduction of nitroblue tetrazolium (NBT; Hi Media, Mumbai, India). The reaction mixture (1 ml) containing 50 mM phosphate buffer (pH 7.0) and 0.1 mM EDTA to which an oxygen-generating system containing 14.3 mM methionine (Hi Media, Mumbai, India), 82.5 µM NBT, and 2.2 µM riboflavin (Hi Media, Mumbai, India), prepared freshly *in situ*, was added. The reaction was initiated by adding 25 µl of crude enzyme. The entire system was kept 30 cm below the light source (six 15W fluorescent tube light) for 30 min. Reaction was stopped by switching off the tube light. For light blank, all the reactants without enzyme extract was incubated in light as for the samples, whereas all the reactants along with 25 µl enzyme extract

were incubated in dark for blank. Reduction in NBT was measured by monitoring the change in absorbance at 560 nm. SOD activity is expressed as µKat of SOD $mg^{-1}$ protein.

**Catalase (CAT) assay (EC 1.11.1.6)**

CAT activity was measured by following the decomposition of hydrogen peroxide ($H_2O_2$) as described by Cakmak and Marschner [17] with minor modifications. The activity was measured in a reaction mixture (1 ml) containing 50 mM phosphate buffer (pH 7.0) and 300 mM $H_2O_2$ (Qualigens, Mumbai, India). The reaction was initiated by adding 50 µl enzyme extract and the activity was determined as a result of $H_2O_2$ decomposition by monitoring the decrease in absorbance at 240 nm ($\varepsilon$ = 36 $mM^{-1}$ $cm^{-1}$) for 2 min at an interval of 15 seconds. The slope of readings between the time interval considered as $\Delta A$ and the enzyme activity was expressed as µ Kat of CAT activity $mg^{-1}$ protein.

**Ascorbate peroxidase (APX) assay (EC 1.11.1.11)**

APX activity was determined according to Nakano and Asada [18]. The reaction mixture (1 ml) contained 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbate and 0.1 mM $H_2O_2$. The reaction was started by adding 50 µl of crude enzyme. Ascorbate oxidation was monitored for 1 min by measuring the decrease in absorbance at 290 nm at every 15 s ($\varepsilon$ = 2.8 $mM^{-1}$ $cm^{-1}$). The enzyme activity was expressed as µKat of APX activity $mg^{-1}$ protein.

**Guaiacol peroxidase (GPX) assay (EC 1.11.1.7)**

GPX activity was assayed according to Hemeda and Klein [19]. The reaction mixture (1 ml) containing 50 mM phosphate buffer (pH 7.0), guaiacol (Hi Media, Mumbai, India), 200 mM $H_2O_2$ and 10 µl enzyme extract. The reaction was started by adding 200 mM $H_2O_2$. The increase in absorbance due to oxidation of guaiacol ($\varepsilon$ = 26.6 $mM^{-1}$ $cm^{-1}$) was monitored at 470 nm. Enzyme activity was expressed as µKat $mg^{-1}$ protein.

**Statistical analysis**

A completely randomized design (CRD) was used in all experiments. The experiments were repeated at least thrice. The data was subjected to analysis of variance (ANOVA) followed by Duncan's multiple range tests (DMRT) at $P<0.05$.

## Results

**Re-emergence studies**

The plants of *P. oleracea* after uprooting from soil were place for a month without watering and exposed to sunlight (maximum intensity 550-600 watt /$m^2$) (Fig.1A, B, C and D). In these plants, the decrease in luster and shrinking of root, stem and leaves was observed during first week of exposure. This might be possible because of water loss, nutrient deficiency and high intensity of sunlight. Subsequently the shrinking in plant parts was prominent and at the end of the second week, the root, stem and leaves were turn gray-brown and blackish in color. Later, in third and fourth week, the plant parts were remain well shrunken and appear grey brown and blackish in color. At the end of the month, the plants were appeared almost dry but the stem portion did not broken on application of pressure. These exposed plants on re-plantation to soil and watering were appeared turgid and emerged after 2–3 days of re-plantation (Fig.1 E and F). These results revealed that the *P. oleracea* has very high water stress and sunlight tolerance capacity. During mechanical removal being small in size, the stem segments may remain on soil which may sprout and develop in to new plants after receiving moisture or watering. Hence, it can be concluded that *P. oleracea* is a serious weed and is difficult to control by conventional methods.

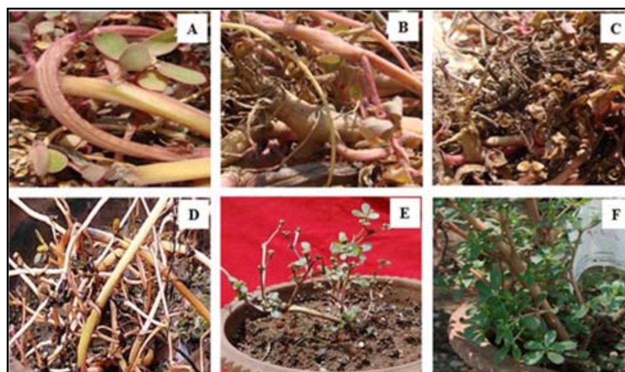


**Fig 1:** Effect of sun exposure and re-emergence in *Portulaca oleracea* L.; Effect of sun exposure (A) 7th day; (B) 14th day; (C) 21st day; (D) 28th day; Re-emergence in plants place without watering after removing from soil and expose to sunlight for 28 days (E) 7th day after re-plantation in soil and watering and (F) 14th day after re-plantation in soil and watering

**Effect of herbicides on chlorophyll content**

Data on effect of different herbicide on chlorophyll content in *P. oleracea* plants grown under natural condition is presented in Table 2. Compare to control, the content of chl a, chl b and total chlorophyll were drastically decreased with treatment of 1% and 5% 2, 4-D. More or less similar decline in the content of chlorophylls was observed with the treatments of other herbicides. Both 1% and 5% treatments of 2, 4-D was highly significant over the similar treatment of Oxyfluorfen and Glyphosate. The results on analysis of chlorophyll content on initiation of treatments and after 1st, 2nd 3rd and 4th day of treatments suggest that the content was decline with increase of time. At the same time, there was no any significant difference observed in the content in control experiments. The effect of Pendimethalin on initiation of treatments and after 1st, 2nd 3rd and 4th day of treatments did not influence the chlorophyll content of *P. oleracea* grown in field conditions. The similar results were recorded in *in vitro* experiments with plants of *P. oleracea* (Table 3).

**Effect of herbicides on Betalain content**

The data recorded in table 4 represents the effect of different herbicide on pigment Betalain- the betacyanin and betaxanthin content in *P. oleracea* grown under field conditions. The data revealed that after initiation of treatment, there was significant increase in both betacyanin and betaxanthin over the control. The higher content of betacyanin (0.38±0.01 mg/g FW) and betaxanthin (0.20±0.02 mg/g) was recorded in the plants treated with 5% 2, 4–D. Similar results were observed with the treatment of Glyphosate. The content of the pigment in plants with the treatment of Pendimethalin at 1% and 5% was comparable to control. The analytical results on 1st, 2nd, 3rd and 4th day of treatments suggest that the content of pigment was decline in plants on 4th day after initiation of the treatment. The results depicted in table 5 suggest that more or less similar trend present for Betalain- the betacyanin and betaxanthin content in control and treated *in vitro* plants of *P. oleracea.*

**Table 2:** Effect of herbicides on the chlorophyll content of *Portulaca oleracea* under field condition.

| Treatment | Day 1 | | | Day 2 | | | Day 3 | | | Day 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Chl-a (mg/g FW) | Chl-b (mg/g FW) | Total chl (mg/g FW) | Chl-a (mg/g FW) | Chl-b (mg/g FW) | Total chl (mg/g FW) | Chl-a (mg/g FW) | Chl-b (mg/g FW) | Total chl (mg/g FW) | Chl-a (mg/g FW) | Chl-b (mg/g FW) | Total chl (mg/g FW) |
| Control | $7.7\pm0.6^{a}$ | $3.3\pm0.4^{a}$ | $11.0\pm1.0^{a}$ | $7.7\pm0.6^{a}$ | $3.2\pm0.4^{a}$ | $10.9\pm1.0^{a}$ | $7.7\pm0.6^{a}$ | $3.0\pm0.4^{a}$ | $10.7\pm1.0^{a}$ | $7.7\pm0.6^{a}$ | $3.0\pm0.3^{a}$ | $10.7\pm0.9^{a}$ |
| 2,4-D 1% | $6.1\pm1.3^{ab}$ | $3.0\pm0.4^{a}$ | $9.1\pm1.7^{ab}$ | $3.1\pm0.5^{de}$ | $1.0\pm0.1^{d}$ | $4.1\pm0.6d^{e}$ | $2.5\pm0.5^{f}$ | $0.5\pm0.3^{e}$ | $3.0\pm0.8^{f}$ | $2.0\pm0.1^{f}$ | $0.5\pm0.1^{d}$ | $2.5\pm0.2^{f}$ |
| Oxyfluorfen 1% | $6.6\pm0.1^{ab}$ | $2.4\pm0.1^{b}$ | $9.0\pm0.2^{ab}$ | $4.8\pm2.1^{bc}$ | $2.1\pm0.8^{b}$ | $6.9\pm2.9b^{c}$ | $3.3\pm0.6^{de}$ | $1.5\pm0.3^{cd}$ | $4.8\pm0.9^{de}$ | $3.0\pm0.3^{e}$ | $1.5\pm0.4^{c}$ | $4.5\pm0.7^{e}$ |
| Glyphosate 1% | $6.1\pm0.6^{ab}$ | $2.2\pm0.5^{bc}$ | $8.3\pm1.1^{ab}$ | $4.0\pm0.5^{cd}$ | $2.2\pm0.3^{b}$ | $6.2\pm0.8^{bcd}$ | $3.9\pm0.5^{cd}$ | $2.0\pm0.2^{bc}$ | $5.9\pm0.8^{cd}$ | $3.8\pm0.4^{d}$ | $2.0\pm0.2^{bc}$ | $5.8\pm0.6^{d}$ |
| Pendimethalin 1% | $5.0\pm0.1^{bc}$ | $1.7\pm0.2^{cde}$ | $6.7\pm0.3^{bc}$ | $4.6\pm1.5^{cd}$ | $1.6\pm0.4^{bcd}$ | $6.2\pm1.8^{bcd}$ | $4.5\pm1.5^{c}$ | $2.3\pm0.1^{ab}$ | $6.8\pm1.6^{c}$ | $5.1\pm0.1^{c}$ | $2.0\pm0.6^{bc}$ | $7.1\pm0.7^{c}$ |
| 2,4-D 5% | $3.7\pm1.9^{c}$ | $1.6\pm0.2^{de}$ | $5.3\pm2.1^{c}$ | $1.9\pm0.2^{e}$ | $1.4\pm0.1^{cd}$ | $3.3\pm0.3^{e}$ | $1.8\pm0.1^{f}$ | $0.9\pm0.1^{de}$ | $2.7\pm0.2^{f}$ | $1.8\pm0.1^{f}$ | $0.5\pm0.2^{d}$ | $2.3\pm0.3^{f}$ |
| Oxyfluorfen 5% | $4.7\pm0.1^{bc}$ | $1.6\pm0.4^{de}$ | $6.3\pm2.8^{bc}$ | $4.0\pm0.1^{cd}$ | $1.9\pm1.0^{bc}$ | $5.9\pm1.1^{cd}$ | $2.4\pm0.2^{ef}$ | $1.8\pm1.0^{bc}$ | $4.2\pm1.2^{ef}$ | $2.0\pm0.2^{f}$ | $1.3\pm0.6^{c}$ | $3.3\pm0.8^{f}$ |
| Glyphosate 5% | $3.6\pm0.3^{c}$ | $1.2\pm0.2^{e}$ | $4.8\pm0.5^{c}$ | $3.2\pm0.2^{de}$ | $2.1\pm0.1^{b}$ | $5.3\pm0.3^{cde}$ | $3.2\pm0.2^{de}$ | $2.1\pm0.2^{bc}$ | $5.3\pm0.4^{cde}$ | $2.6\pm0.2^{e}$ | $2.0\pm0.3^{bc}$ | $4.6\pm0.5^{e}$ |
| Pendimethalin 5 % | $4.7\pm1.8^{bc}$ | $2.0\pm0.2^{bcd}$ | $6.7\pm2.0^{bc}$ | $6.3\pm0.1^{b}$ | $2.1\pm0.2^{b}$ | $8.4\pm0.3^{b}$ | $6.3\pm0.1^{b}$ | $2.2\pm0.3^{bc}$ | $8.5\pm0.4^{b}$ | $6.5\pm0.2^{b}$ | $2.3\pm0.4^{b}$ | $8.8\pm0.6^{b}$ |

**Table 3:** Effect of herbicides on the chlorophyll content of *Portulaca oleracea* under *in vitro* condition.

| Treatment | Day 1 | | | Day 2 | | | Day 3 | | | Day 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Chl-a (mg/g FW) | Chl-b (mg/g FW) | Total chl (mg/g FW) | Chl-a (mg/g FW) | Chl-b (mg/g FW) | Total chl (mg/g FW) | Chl-a (mg/g FW) | Chl-b (mg/g FW) | Total chl (mg/g FW) | Chl-a (mg/g FW) | Chl-b (mg/g FW) | Total chl (mg/g FW) |
| Control | $5.3\pm0.4^{a}$ | $1.6\pm0.1^{a}$ | $6.9\pm0.5^{a}$ | $5.1\pm0.4^{a}$ | $1.5\pm0.1^{a}$ | $6.6\pm0.5^{a}$ | $5.1\pm0.4^{a}$ | $1.6\pm0.1^{a}$ | $6.7\pm0.5^{a}$ | $5.2\pm0.4^{a}$ | $1.6\pm0.1^{b}$ | $6.8\pm0.5^{a}$ |
| 2,4-D 1% | $3.4\pm0.2^{c}$ | $1.0\pm0.1^{d}$ | $4.5\pm0.3^{b}$ | $3.2\pm0.3^{cd}$ | $1.0\pm0.2^{b}$ | $4.2\pm0.5^{c}$ | $3.0\pm0.2^{c}$ | $0.8\pm0.2^{c}$ | $3.9\pm0.4^{bc}$ | $1.4\pm0.1^{e}$ | $0.5\pm0.1^{c}$ | $1.8\pm0.2^{c}$ |
| Oxyfluorfen 1% | $2.8\pm0.2^{cde}$ | $0.7\pm0.1^{e}$ | $3.5\pm0.3^{cde}$ | $2.8\pm0.1^{de}$ | $0.5\pm0.1^{c}$ | $3.3\pm0.2^{de}$ | $2.6\pm0.1^{cd}$ | $0.4\pm0.1^{d}$ | $3.1\pm0.2^{de}$ | $2.1\pm0.1^{d}$ | $0.4\pm0.1^{cd}$ | $2.5\pm0.2^{bc}$ |
| Glyphosate 1% | $3.2\pm0.1^{cd}$ | $1.0\pm0.1^{e}$ | $4.2\pm0.2^{bc}$ | $3.1\pm0.1^{cd}$ | $0.9\pm0.1^{b}$ | $4.0\pm0.2^{cd}$ | $3.0\pm0.1^{c}$ | $0.7\pm0.1^{c}$ | $3.7\pm0.2^{cd}$ | $2.7\pm0.1^{c}$ | $0.4\pm0.1^{cd}$ | $3.0\pm0.2^{b}$ |
| Pendimethalin 1% | $4.2\pm0.9^{b}$ | $1.4\pm0.1^{b}$ | $4.2\pm1.0^{bc}$ | $4.2\pm0.9^{b}$ | $1.5\pm0.1^{a}$ | $5.7\pm1.0^{b}$ | $4.4\pm0.6^{b}$ | $1.6\pm0.1^{a}$ | $4.6\pm0.7^{b}$ | $4.4\pm0.6^{b}$ | $2.2\pm0.3^{a}$ | $6.6\pm0.9^{a}$ |
| 2,4-D 5% | $1.6\pm0.1^{f}$ | $1.2\pm0.1^{c}$ | $2.7\pm0.2^{f}$ | $1.5\pm0.1^{f}$ | $1.0\pm0.1^{b}$ | $2.5\pm0.2^{f}$ | $1.3\pm0.1^{e}$ | $1.0\pm0.1^{b}$ | $2.3\pm0.2^{f}$ | $0.5\pm0.1^{f}$ | $0.2\pm0.1^{d}$ | $0.7\pm0.2^{d}$ |
| Oxyfluorfen 5% | $2.3\pm0.1^{e}$ | $0.7\pm0.1^{e}$ | $3.0\pm0.2^{ef}$ | $2.3\pm0.1^{e}$ | $0.6\pm0.1^{c}$ | $2.9\pm0.2^{e}$ | $2.1\pm0.1^{d}$ | $0.6\pm0.1^{cd}$ | $2.6\pm0.2^{ef}$ | $1.5\pm0.1^{e}$ | $0.4\pm0.1^{cd}$ | $2.0\pm0.2^{c}$ |
| Glyphosate 5% | $2.6\pm0.1^{de}$ | $0.7\pm0.1^{e}$ | $3.3\pm0.2^{def}$ | $2.6\pm0.1^{de}$ | $0.6\pm0.1^{c}$ | $3.2\pm0.2^{de}$ | $2.5\pm0.1^{cd}$ | $0.6\pm0.1^{cd}$ | $3.1\pm0.2^{de}$ | $2.1\pm0.1^{d}$ | $0.3\pm0.1^{cd}$ | $2.4\pm0.2^{bc}$ |
| Pendimethalin 5 % | $3.4\pm0.2^{c}$ | $1.1\pm0.1^{e}$ | $4.0\pm0.3^{bcd}$ | $3.5\pm0.2^{c}$ | $1.3\pm0.1^{a}$ | $4.2\pm0.3^{c}$ | $4.5\pm0.5^{b}$ | $1.4\pm0.1^{a}$ | $4.4\pm0.6^{bc}$ | $4.5\pm0.5^{b}$ | $1.8\pm0.2^{b}$ | $6.3\pm0.7^{a}$ |

**Table 4:** Effect of herbicides on the pigment content of *Portulaca oleracea* under field condition.

| Treatment | Day 1 | | Day 2 | | Day 3 | | Day 4 | |
|---|---|---|---|---|---|---|---|---|
| | Betacyanin (mg/g FW) | Betaxanthin (mg/g FW) | Betacyanin (mg/g FW) | Betaxanthin (mg/g FW) | Betacyanin (mg/g FW) | Betaxanthin (mg/g FW) | Betacyanin (mg/g FW) | Betaxanthin (mg/g FW) |
| Control | $0.22\pm0.02^{c}$ | $0.11\pm0.01^{e}$ | $0.23\pm0.02^{c}$ | $0.11\pm0.01^{d}$ | $0.21\pm0.02^{bc}$ | $0.18\pm0.01^{a}$ | $0.22\pm0.02^{b}$ | $0.18\pm0.01^{ab}$ |
| 2,4-D 1% | $0.36\pm0.01^{a}$ | $0.17\pm0.01^{cd}$ | $0.29\pm0.01^{a}$ | $0.13\pm0.01^{bc}$ | $0.25\pm0.01^{ab}$ | $0.11\pm0.01^{bc}$ | $0.11\pm0.01^{de}$ | $0.05\pm0.01^{b}$ |
| Oxyfluorfen 1% | $0.28\pm0.02^{b}$ | $0.20\pm0.01^{ab}$ | $0.26\pm0.01^{abc}$ | $0.14\pm0.01^{b}$ | $0.22\pm0.01^{bc}$ | $0.11\pm0.01^{bc}$ | $0.12\pm0.01^{d}$ | $0.07\pm0.01^{ab}$ |
| Glyphosate 1% | $0.35\pm0.03^{a}$ | $0.19\pm0.01^{bc}$ | $0.25\pm0.01^{bc}$ | $0.14\pm0.01^{b}$ | $0.24\pm0.02^{b}$ | $0.11\pm0.01^{bc}$ | $0.16\pm0.01^{c}$ | $0.09\pm0.01^{a}$ |
| Pendimethalin 1% | $0.25\pm0.01^{b}$ | $0.20\pm0.01^{ab}$ | $0.28\pm0.03^{ab}$ | $0.16\pm0.02^{a}$ | $0.29\pm0.03^{a}$ | $0.17\pm0.02^{a}$ | $0.29\pm0.03^{a}$ | $0.17\pm0.02^{ab}$ |
| 2,4-D 5% | $0.38\pm0.01^{a}$ | $0.20\pm0.02^{ab}$ | $0.23\pm0.01^{c}$ | $0.12\pm0.01^{cd}$ | $0.18\pm0.01^{c}$ | $0.09\pm0.01^{c}$ | $0.08\pm0.01^{f}$ | $0.04\pm0.01^{b}$ |
| Oxyfluorfen 5% | $0.29\pm0.06^{b}$ | $0.19\pm0.01^{bc}$ | $0.19\pm0.03^{d}$ | $0.11\pm0.01^{d}$ | $0.19\pm0.03^{c}$ | $0.11\pm0.01^{bc}$ | $0.09\pm0.01^{ef}$ | $0.07\pm0.01^{ab}$ |
| Glyphosate 5% | $0.37\pm0.03^{a}$ | $0.22\pm0.03^{a}$ | $0.23\pm0.01^{c}$ | $0.14\pm0.01^{b}$ | $0.22\pm0.01^{bc}$ | $0.12\pm0.01^{b}$ | $0.13\pm0.01^{d}$ | $0.10\pm0.01^{ab}$ |
| Pendimethalin 5 % | $0.23\pm0.01^{c}$ | $0.16\pm0.01^{d}$ | $0.26\pm0.01^{abc}$ | $0.16\pm0.01^{a}$ | $0.27\pm0.01^{bc}$ | $0.16\pm0.01^{a}$ | $0.27\pm0.01^{a}$ | $0.16\pm0.01^{ab}$ |

**Table 5:** Effect of herbicides on the pigment content of *Portulaca oleracea* under *in vitro* condition.

| Treatment | Day 1 | | Day 2 | | Day 3 | | Day 4 | |
|---|---|---|---|---|---|---|---|---|
| | Betacyanin (mg/g FW) | Betaxanthin (mg/g FW) | Betacyanin (mg/g FW) | Betaxanthin (mg/g FW) | Betacyanin (mg/g FW) | Betaxanthin (mg/g FW) | Betacyanin (mg/g FW) | Betaxanthin (mg/g FW) |
| Control | $0.26\pm0.01^{e}$ | $0.16\pm0.01^{cd}$ | $0.27\pm0.01^{ab}$ | $0.16\pm0.01^{c}$ | $0.27\pm0.03^{b}$ | $0.16\pm0.01^{c}$ | $0.29\pm0.03^{a}$ | $0.19\pm0.01^{b}$ |
| 2,4-D 1% | $0.31\pm0.01^{b}$ | $0.17\pm0.02^{c}$ | $0.26\pm0.01^{bc}$ | $0.15\pm0.02^{cd}$ | $0.15\pm0.01^{cd}$ | $0.12\pm0.02^{e}$ | $0.09\pm0.01^{cd}$ | $0.08\pm0.01^{de}$ |
| Oxyfluorfen 1% | $0.27\pm0.01^{de}$ | $0.15\pm0.01^{de}$ | $0.21\pm0.01^{f}$ | $0.13\pm0.01^{d}$ | $0.15\pm0.01^{cd}$ | $0.10\pm0.01^{f}$ | $0.12\pm0.01^{bc}$ | $0.08\pm0.01^{de}$ |
| Glyphosate 1% | $0.26\pm0.01^{e}$ | $0.14\pm0.02^{e}$ | $0.23\pm0.02^{def}$ | $0.13\pm0.01^{d}$ | $0.16\pm0.01^{c}$ | $0.11\pm0.02^{ef}$ | $0.13\pm0.01^{b}$ | $0.10\pm0.01^{cd}$ |
| Pendimethalin 1 % | $0.29\pm0.01^{c}$ | $0.22\pm0.01^{a}$ | $0.25\pm0.01^{bcd}$ | $0.21\pm0.01^{a}$ | $0.30\pm0.02^{a}$ | $0.22\pm0.01^{a}$ | $0.29\pm0.03^{a}$ | $0.22\pm0.01^{a}$ |
| 2,4-D 5% | $0.33\pm0.01^{a}$ | $0.16\pm0.02^{cd}$ | $0.29\pm0.01^{a}$ | $0.13\pm0.01^{d}$ | $0.13\pm0.01^{d}$ | $0.10\pm0.01^{f}$ | $0.07\pm0.01^{d}$ | $0.06\pm0.01^{e}$ |
| Oxyfluorfen 5% | $0.28\pm0.01^{cd}$ | $0.16\pm0.01^{cd}$ | $0.22\pm0.01^{ef}$ | $0.13\pm0.01^{d}$ | $0.15\pm0.01^{cd}$ | $0.12\pm0.01^{e}$ | $0.11\pm0.01^{bc}$ | $0.10\pm0.01^{cd}$ |
| Glyphosate 5% | $0.27\pm0.01^{de}$ | $0.17\pm0.01^{c}$ | $0.24\pm0.01^{cde}$ | $0.16\pm0.01^{c}$ | $0.16\pm0.01^{c}$ | $0.14\pm0.01^{d}$ | $0.12\pm0.01^{bc}$ | $0.11\pm0.01^{c}$ |
| Pendimethalin 5 % | $0.28\pm0.01^{cd}$ | $0.19\pm0.01^{b}$ | $0.27\pm0.01^{ab}$ | $0.19\pm0.02^{b}$ | $0.26\pm0.01^{b}$ | $0.19\pm0.03^{b}$ | $0.27\pm0.01^{a}$ | $0.19\pm0.03^{b}$ |

### Effect of herbicides on Enzyme analysis

Results on activity of enzyme superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guiacol peroxidase (GPX) in control and herbicide treated plants depicted in Fig. 3 and 4. Revealed that in both *in vitro* and plants grown in natural condition, the activity was higher with the treatment of 2, 4-D and Oxyfluorfen during the 1st and 2nd day after initiation of the treatments (Fig. 3 and 4). While the enzyme activity was decline in both type of treated plants during the 3rd and 4th day. The minimum APX activity (0.84±0.06 μKat/mg protein) was observed with 1% treatment of 2,4–D to the plants grown in natural condition whereas the activity was minimum (1.54±0.01 μKat/mg protein) in *in vitro* grown plants with the 5% treatment of 2, 4–D. The GPX activity (5.74±0.65 μKat/mg protein) was maximum during the first day of initiation of treatment with 1% 2, 4–D. The decline in activity was observed with the 5% treatment of 2, 4–D. Similar trend was observed for the activity of CAT and SOD. The results were more or less similar for enzyme activity in *in vitro* grown plants of *P. oleracea* (Fig. 4).

**Fig 2:** Effect of herbicides on *Portulaca oleracea* under field condition. (a) Control (b) 1% 2,4-D; (c) 5%2,4-D; (d) 1% Oxyfluorfen; (e) 5% Oxyfluorfen; (f) 1% Glyphosate (g) 5% Glyphosate; (h) 1% Pendimethalin (i) 5%Pendimethalin

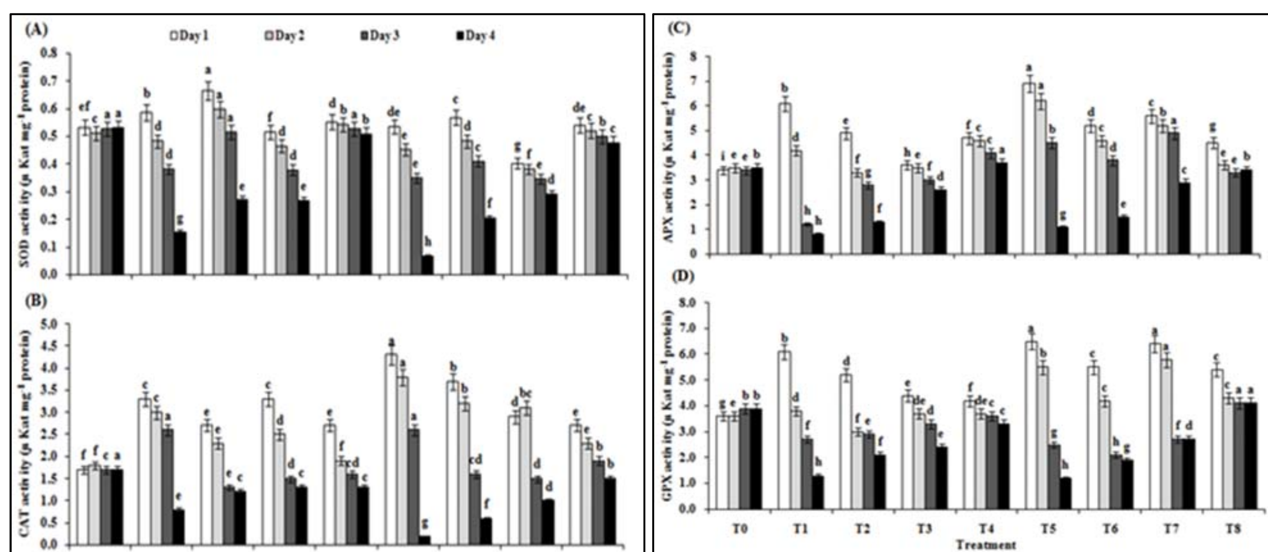


**Fig 3:** Antioxidant enzymes (CAT, APX, GPX and SOD) activity in *Portulaca olaracea.* Field grown plants- T0: Control; T1: 1% 2, 4-D; T2: 1% Oxyfluorfen; T3: 1% Glyphosate; T4: 1% Pendimethalin; T5: 5% 2, 4-D; T6: 5% Oxyfluorfen; T7: 5% Glyphosate and T8: 5% Pendimethalin. Each value represents mean of three replications and vertical bars indicate SE. The bars with different letters on the same color columns are significantly different at $P<0.05$

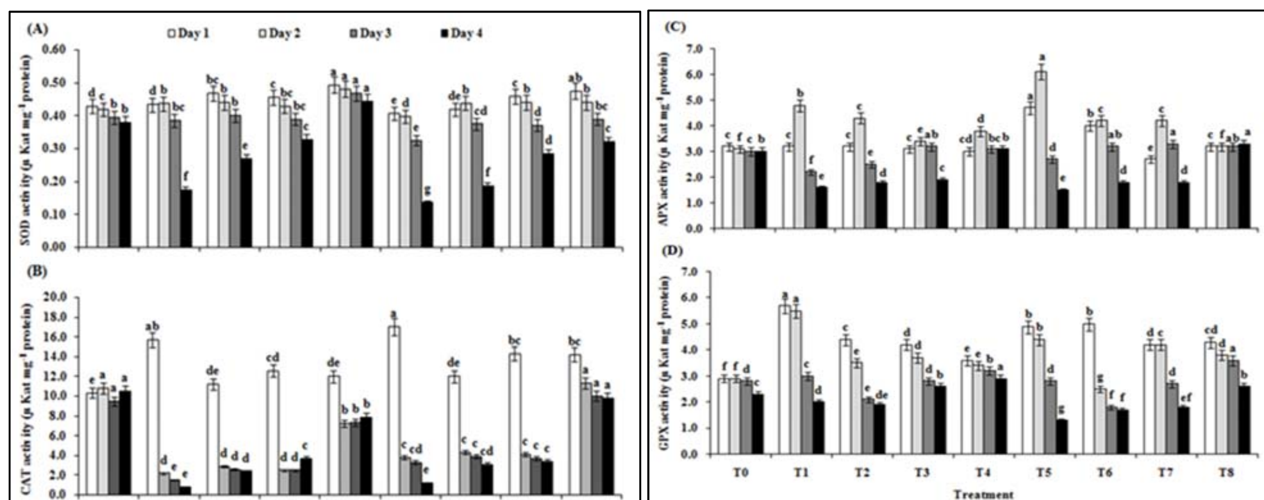


**Fig 4:** Antioxidant enzymes (CAT, APX, GPX and SOD) activity in *Portulaca olaracea*. *In vitro* grown shoots- T0: Control; T1: 1% 2, 4-D; T2: 1% Oxyfluorfen; T3: 1% Glyphosate; T4: 1% Pendimethalin; T5: 5% 2, 4-D; T6: 5% Oxyfluorfen; T7: 5% Glyphosate and T8: 5% Pendimethalin. Each value represents mean of three replications and vertical bars indicate SE. The bars with different letters on the same color columns are significantly different at P<0.05

**Discussion**

The weeds are an undesired plant that needs to be eradicating from the field. In most cases the uprooted weeds easy to collect and thrown out of field, which also wilt rapidly and died. However, this is not the case for *P. oleracea.* The characteristic weedy nature of *P. oleracea* is attributed to its ability to produce as many as 10000 seeds, easy dispersal, and rapid growth, competitive with crops for space, light, water and nutrients and possess little recognized value [3]. Generally traditional methods of manual hoeing or tractor drawn cultivator are used for its removal or control. However, with these methods removal of all the seeds and stem segments is highly difficult and tedious task due to their small size and easy propagation through the seeds and stem segments. In addition it is highly salt and drought tolerant [5, 6]. In the present investigation, the *P. oleracea* plants after removing from soil were place for a month without watering and exposed to sunlight (maximum intensity 550-600 watt/m$^2$). These exposed plants showed the rapid re–emergence after plantation in soil and watering. The adaption of C4 photosynthesis, succulence nature in plant parts and higher regenerative ability in stem allows its survival in difficult situations. These results confirm that *P. oleracea* is highly difficult to eradicate from the field by conventional methods. Under such circumstances, an herbicidal chemical finds the importance.

Several herbicidal chemicals with different composition and spectrum are available in market. Lot many studies on weed control using chemical herbicide have been documented [11, 20]. In certain cases, like *P. oleracea* the studies on physiological and biochemical changes with herbicide treatment are limited. The results of the present study on pigments and enzyme activity helps to understand the prolong survival strategy of *P. oleracea* under adverse conditions especially with the treatment of herbicide Oxyfluorfen, Glyphosate, Pendimethalin and 2, 4-D. The experiments with treatment of 1% and 5% concentrations of the herbicide and subsequent observations for about five days for pigment content and enzyme activity revealed that 2, 4-D and oxyfluorfen were effective to control the weed *P. oleracea*. On the day of herbicide application, there was no visible effect but from day two the effects were observable. The bleaching of leaves was observed with the treatment of 5% 2, 4-D while the loss of leaf shine was observed with treatment of 5% oxyfluorfen. Compare to control, the content of chla, chlb and total chlorophyll were drastically decreased with treatment of 1% and 5% 2, 4-D. More or less similar decline in the content of chlorophylls was observed with the treatments of other herbicides in earlier reports, the application of herbicide 2, 4–D resulted in effective control of several weeds [11, 21, 22]. The 2, 4–D effects growth pattern of plant, shoot and root elongation and parenchymatous cells [23, 24]. It induces mitotic aberrations, inhibits cell enlargement, differentiation and division [25, 26]. The increased respiration and depletion of food reserves might be the possible mechanism responsible for herbicidal action of 2, 4-D. The isozyme oxidase acts on 2, 4-D and produce phenols which on accumulation may inhibit plant growth [27, 28]. The application of herbicide oxyfluorfen through contact action cause the membrane disruption, inhibit photosynthetic electron transport and ATP synthesis [29]. The membrane disruption property may not allow establishment of proton gradient across the chloroplast and mitochondrial membrane which is essential activity for ATP synthesis [30]. The another reason of the effectiveness of oxyfluoren is it stops chlorophyll biosynthesis severely by inhibiting alteration route of Protoporphyrinogen IX to Protoporphyrin IX which is essential for chlorophyll synthesis [31].

Glyphosate is widely used to kill unwanted plants both in agriculture and non-agricultural fields. The earlier reports suggest that it inhibits the enzymes of the shikimic acid pathway and preventing plants from synthesizing essential three aromatic amino acids. The absence of these amino acids affect the growth and survival of most plants. In addition it also affects other enzymes as in sugar cane; it reduces the activity of the enzyme related to sugar metabolism. Pendimethalin is having herbicideal action by inhibition of cell division and cell elongation at pre-emergence and post-emergence stages of development of grasses and certain broad leaf weeds.

The involvement of herbicide 2, 4-D and Oxyfluorfen directly in the cell division, membrane disruption and pigment synthesis might be the reasons why 2,4-D and oxyfluorfen were effective to control the growth and cause the death of *P. oleracea*. The role of Glyphosate and Pendimethalin is limited to the certain part of metabolism and this may be the reason

that they are not highly effective to control the *P. oleracea.* Thus the variable response of *P. oleracea* to the treatment of herbicides Oxyfluorfen, Glyphosate, Pendimethalin and 2, 4-D is in accordance with the earlier reports that the effective response of chemical as widicide varies with the type of plant. Glyphosate reduced barley shoot delta-aminolevulinic acid synthesis and drastically decreased chlorophyll content [32]. Chlorophyll a and b content, net photosynthesis rate and the relative rate of electron transport was decreased significantly after treated with sprays of glyphosate in *Imperata cylindrica* L. (cogongrass) [33]. Chlorophyll b content decreased after application of Pursuit herbicide to weeds *Trifolium pretense* L. and *Trifolium alexandrinum* L [34]. Application of herbicide resulted in significant decline of total chlorophyll content in *Trianthema portulacastrum* L [35]. Spraying of herbicide onto the weed effects essential photosynthetic pigment, chlorophyll and disturbs the anti-oxidative enzymes function which leads to collapse of defense mechanism of weed [31].

*Portulaca oleracea* is characterized by the presence of colored pigments called betalains which are classified into two main categories: betaxanthin and betacyanin. Betaxanthin represent yellow-orange pigments, usually existing in flowers while betacyanin are the red to violet pigments typically observed in vegetative plant parts and are dominant under stress. In the present investigation, on application of different herbicides the content of betalain during initial days of treatment was higher over the control; this may be possible due stress caused by the herbicide. Usually, the increase in flavonoids, anthocyanins, carotenoids and ascorbic acid appeared as a protective metabolites under stress conditions [36]. However, during subsequent days the content was decline. This may be attributed to the participation and inhibition of herbicide in biosynthetic pathways of the plant.

It is well documented that under stressful situations, the appearance of free radicals is very common which results in oxidative damage. To prevent the damage, the major role is played by the free radical scavenging enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX), glutathione reductase (GR) and mono dehydroascorbate reductase (MDAR) [36]. In present study the free radical scavenging enzymes activity initially in the treated plants were comparatively higher over the control, later the activity decline. The increase could be the result of induction of antioxidant system under stress but later decline may be due to the disturbance cause in structure and metabolism by the action of herbicides.

The trend of activity of these antioxidant enzymes remains more or less same for most of the herbicides treatments [37]. Similar increase in activity of SOD, and CAT recorded in crop associated weeds *Ambrosia artemisifolia* L., *Chenopodium album* L., *Convolvulus arvensis* L. and *Sinapis arvensis* L. after exposure to herbicides linuron and dimethenamid [38]. In accordance, significant increase in activities of SOD, CAT, APX and GPX was recorded on application of methyl viologen herbicide to *Azolla microphylla* [39]. The treatment of herbicide glyphosate promotes the activity of SOD, CAT and APX in *Arabidopsis thaliana* [40].

In crop plant *Avena sativa, Brassica campestris* L. cv. *chinensis* and *Lactuca sativa* after exposure to herbicide sodium trichloroacetate, similar increase in activity of SOD, CAT, APX and GPX recorded [41]. After treatment of weedicide isoproturon in maize, initial increase and later decline in activity of these antioxidant enzyme was observed [11, 42]. The results on *in vitro* experiments were also similar and that confirms influence of these herbicide on *P. oleracea*. In accordance, the elevation of the free radical scavenging enzymes has been positively correlated with abiotic stress tolerance and the same is also true for *in vitro* cultures [43]. Therefore, the results of the present investigation and earlier reports suggest that a speedy preliminary screening of herbicide effect is possible by assessing biochemical and physiological activities in *in vitro* cultures.

**Conclusion**

In light of the results obtained from this investigation, it is revealed that herbicide 2, 4-D and Oxyfluorfen treatments caused inhibitory effect on pigments chlorophyll and betalain content and enzyme activity in both *in vitro* cultures and plants grown in natural conditions. Both herbicides were phytotoxic and effective for termination of growth. The results provide evidence that *P. oleracea* has strong ability to survive in adverse conditions and 2, 4-D and oxyfluorfen can be effective herbicide to control world's most unscrupulous weed.

**Acknowledgment**

The authors wish to acknowledge the financial support from the Department of Botany, Savitribai Phule Pune University, Pune, under UGC SAP-DRS-III, DST- FIST, DST-PURSE program of Government of India. Author LS is thankful to Savitribai Phule Pune University, Pune for providing the stipend.

**Compliance with ethical standards**

Conflict of interest L. Shanmugam, M. R. Chambhare, S. Kadlag, M. L. Ahire and T. D. Nikam declare that they have no conflict of interest.

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