MATERIALS AND METHODS
The plant selected for the present study is *Psoralea corylifolia* L., variety PS-11062 commonly known as Babchi. The healthy and authentic seeds were obtained from National Biological Plant and Genome research (NBPG), Indian Agricultural Research Institute, New Delhi.

**PLANT DESCRIPTION**

*Psoralea* is a climbing variety of bean commonly found all over China. *Psoralea* seeds are large, hard and black in color. These are dissimilar to the common variety of beans grown in the gardens; the seeds of *Psoralea* are heady and bitter to taste. The seeds are normally harvested during the fall when they are ripe. A herbaceous annual shrub grows up to one meter in height. The leaves, arranged in racemes, are simple and broadly elliptic. On both the surfaces, the leaves are clothed with white hairs. The flowers are blue in color, axillary in 10-30 flowered racemes. The seeds are flat closely pitted and black. The seeds are dark brown and elongated. The glabrous closely pitted seeds are compressed, non endospermic, smooth, oily and free of starch. The plant flowers from August to December. The *Psoralea* herb grows and thrives well in any average garden top soil. The plant however, prefers a well-drained soil and enough of sunlight.

**Habitat and Cultivation**

*Psoralea* grows throughout India, especially in the plains of Central and Eastern India, in abundance. The *Psoralea* plants are very sensitive in the sense that they
cannot endure any disturbance of the root and hence it is advisable while the plants are still small they should be planted outdoors in their stable place. The Psoralea enjoys a symbiotic or ‘give-and-take’ rapport with specific bacteria in the soil. It flowers from July to August, and the seeds ripen from September to October. The flowers are hermaphrodite and are pollinated by insects. It can fix nitrogen. The plant prefers light (sandy), medium (loamy) and heavy (clay) well-drained soils.

Medicinal Value
In China and Japan, Psoralea is among the key herbs used extensively by herbalists to treat the various skin disorders. Chinese and Japanese herbal medicine practitioners have been using the herb to cure eczema, vitiligo as well as hair loss. Besides its benefits in treating skin disorder, Psoralea combats bacterial infections and at the same time, invigorates the heart. Psoralen is known as buguzhi that literally means ‘tonify bone resin’. The herb is useful for enhancing bone calcification or adding calcium and therefore it is valuable in osteoporosis and bone fractures. Psoralea comprises of a substance called psoralen which enhance pigmentation of the skin and hence useful in treating vitiligo. At the same time, psoralens improve the flow of blood and enhance the activity to produce melanin (pigments) on the unhealthy areas of the skin.

Researches have shown that the Psoralea plant contain elements such as bavachinin, corylifolinin and psoralen which are responsible in slowing down the speedy growth of osteosarcoma as well as lung cancer cells.

CHEMICALS USED
All the chemicals used in this study were of GR or AR quality. Major and minor salts, buffer components, etc. were purchased from E. MERCK and SRL. Most of the biochemicals used were purchased from Sigma Aldrich (St. Louis Mo, USA).
EXPERIMENTAL SET UP

Procurement of seeds and irradiation

The seeds of Babchi (*Psoralea corylifolia* L.) were procured from NBPR (Indian Agriculture Research Institute, New Delhi. Each sample of 25g of seeds was packed in zipknots and were irradiated with different doses of gamma rays (T₁ - 2.5, T₂ - 5, T₃ - 10, T₄ - 15 and T₅ - 20 kGy) at dose rate of 1.65kGyh⁻¹(Fricke and Hart, 1996) at room temperature (25 ± 1°C) from $^{60}$Co (Gamma chamber, GC, 5000) at INMAS (Institute of Nuclear Medicine and Applied Sciences), New Delhi.

Field experiments were repeated three times with ten replicates. All the seeds were planted in an open atmosphere of Hamdard University, New Delhi, India (28° 38'N, 77° 11'E; elevation of 228 m), about 70-80% relative humidity, 35°C temperature, sandy-loam soil (pH 7.3). The levels of available nitrogen and sulphur in the soil were 51 ppm and 7.9 ppm respectively. Seeds were sown in the first week of April 2008. Each sample were planted in six rows, 4m long and 0.6m wide, making an area of 14.4m². Hills were 30cm apart; with five seeds per hill to avoid root disturbance as *Psoralea* plants are very sensitive in the sense that they cannot endure any disturbance of the root. Other agricultural practices such as irrigation and weeding were carried out as required. The planted seeds were observed daily until germination commenced. The dates of commencement and termination of germination as well as the number of seeds that germinated each day were noted for each sample. The seedlings were collected at three phenological stages (preflowering (45 days after sowing, DAS), flowering (90 DAS) and postflowering (135 DAS)) to analyze the effects of the gamma irradiation on vegetative, reproductive traits and psoralen content in *Psoralea corylifolia* with reference to variable doses of gamma rays.
METHODOLOGY FOLLOWED

Experiment 1. Variation in germination percentage of *Psoralea corylifolia* L. exposed to different doses of gamma rays under field and laboratory conditions.

**Under lab conditions:** A random lot of 200 irradiated seeds for each level of radiation and untreated seeds (control) was grown between two layers of filter paper, kept constantly moist with distilled water in sterile corning glass petri dishes during first week of April 2008. Each treatment has ten replicates and percentage of seed germination was recorded. Emergence of radical was taken as the criterion of germination.

**Under field Conditions:** Another random lot of 200 irradiated seeds for each level of irradiation and untreated seeds (control) were sown in earthen pots of 22cm diameter, filled with homogenously manured garden soil. Ten replicates were taken for each treatment. The sowing was done during first week of April 2008 and care was taken to place the seeds at almost equal depth. Emergence of cotyledons above the soil surface was taken as criterion for germination, and the percentage of seed germination was recorded.

Experiment 2. Variation in growth and morphological characteristics of *Psoralea corylifolia* L. exposed to different doses of gamma rays under field and laboratory conditions.

*Morphological parameters*

Samples were collected at 45-day interval and following growth criteria were recorded, vegetative traits like plant height (cm), plant biomass (g), number of branches and leaves per plant, leaf area per plant (cm²) and reproductive traits like number of raceme per plant, number of flowers per raceme and number of seeds per raceme were analyzed and compared to control samples (Callan and
Kennedy, 1996). The area of leaves was measured in cm\(^2\) by using a leaf area meter (3000A, LiCor, U.S.A). Following morphological parameters were studied:

- Root length
- Shoot length
- Plant height
- Root:Shoot ratio
- Number of branches
- Number of leaves per plant
- Total leaf area
- Root dry weight
- Stem dry weight
- Root:Stem dry weight ratio
- Leaf dry weight
- Total Plant biomass
- Number of racemes per plant
- Number of flowers per raceme
- Number of seeds per raceme
- Number of seeds per plant
- Weight of 100 seeds from the last experiment.

**Plant biomass measurement**

Each plant was separated into leaves, roots, stems and petioles. The plant parts were dried at 55°C for 48 h in Precision™ mechanical convection oven (GCA Corporation) and weighed with Mettler™ balance (type H6), which is accurate upto four decimal places. Portions of each plant were weighed separately to see if there was a change in dry matter accumulation within whole plant. Soil was washed from roots of each plant. The roots were dried and weighed.
Experiment 3. Variation in pigment concentration of *P. corylifolia* L. exposed to different doses of gamma rays.

The fresh leaves were collected from *P. corylifolia* L. plants at the three developmental stages: Preflowering, flowering and postflowering stages i.e. 45, 90 and 135 DAS respectively to investigate variation in the following pigment concentration:

- Chlorophyll 'a'
- Chlorophyll 'b'
- Total chlorophyll
- Carotenoid

**Procedure:** Hiscox and Israelstams (1979) method was used to estimate the pigment concentration in the samples. The method involves the estimation of plant pigments without maceration. Leaves were kept on a moist filter paper in an icebox, washed with cold distilled water and then chopped. 100 mg of the chopped leaf material was taken in vials in triplicates containing 7 ml of dimethyl sulfoxide (DMSO). The vials were then kept in an oven at 65°C for 1 hr for complete leaching of the pigments. Thereafter, the volume of DMSO was made up to 10 ml. The chlorophyll content was then measured immediately. The absorbance of DMSO containing pigments was recorded at 480, 510, 645 and 663 nm using a Beckman spectrophotometer (model DU 640, Fullerton, USA).

**Estimation of plant pigments:** Values of optical densities (ODs) were used to compute the chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids contents using the following formulae given by Maclachlan and Zalik (1963) for chlorophyll *a*, Duxbury and Yentsch (1956) for chlorophyll *b*, Arnon (1949) for total chlorophyll and Barnes et al. (1992) for carotenoids.
Material and Methods

\[
Chl'a'(mg\ g^{-1}\ fr.\ wt.) = \frac{[12.3(OD_{663}) - 0.86(OD_{645})]}{(D \times 1000 \times W)} \times V
\]

\[
Chl'b'(mg\ g^{-1}\ fr.\ wt.) = \frac{[19.3(OD_{645}) - 3.60(OD_{663})]}{(D \times 1000 \times W)} \times V
\]

\[
Total\ 'Chl'(mg\ g^{-1}\ fr.\ wt.) = \frac{[20.2(OD_{645}) + 0.02(OD_{663})]}{(D \times 1000 \times W)} \times V
\]

\[
Carotenoid\ (mg\ g^{-1}\ fr.\ wt.) = \frac{[7.6(OD_{480}) - 1.49(OD_{510})]}{(D \times 1000 \times W)} \times V
\]

Where,

- \(D\) = Distance travelled by the light path
- \(W\) = Weight of the leaf material taken
- \(V\) = Volume of the extract
- \(OD\) = Optical density

Experiment 4. Variation in biochemical aspects of \textit{Psoralea corylifolia} L. exposed to different doses of gamma rays.

The fresh leaves were collected from \textit{P. corylifolia} L. plants at three developmental stages i.e. Preflowering, flowering and postflowering stages (45, 90, 145 DAS) to investigate variation in following biochemical aspects.

- Soluble protein content
- Soluble amino acids
- Nitrate content
- Nitrate reductase activity
- Soluble sugar
- Proline content

A. Soluble protein content

The total soluble protein content of different sample was estimated following the method of Bradford (1976).
Preparation of reagents

i. Extraction buffer:
0.1 M phosphate buffer (pH 7.0) was used as extraction buffer. The solution of sodium dihydrogen phosphate (NaH$_2$PO$_4$) and disodium hydrogen phosphate (Na$_2$HPO$_4$) was prepared in the following manner:

Solution A: 1.42 g of Na$_2$HPO$_4$ was dissolved in DDW and the volume was made to 100 ml.

Solution B: 0.78 g of NaH$_2$PO$_4$ was dissolved in DDW and the volume was made to 50 ml.

Solution A and B were mixed in an appropriate ratio to adjust the pH at 7.0.

ii. 10% (w/v) TCA
10 g of TCA was dissolved in doubled distilled water to a final volume of 100 ml.

iii. 0.1 N NaOH
0.4 g of sodium hydroxide (NaOH) pellets was dissolved in double distilled water to make a final volume of 100 ml.

iv. Bradford’s reagent
50 ml of 90% ethanol was mixed to 100 ml of orthophosphoric acid (85%). Its volume was made up to 1 L and 100 mg of Coomassie Brilliant Blue (G) dye was added to it which was stirred well on a magnetic stirrer in dark covered volumetric flask. The solution was then filtered through Whatman filter paper No.1 and stored in dark conditions. The final concentrations of components in the reagent were 0.01% Coomassie brilliant blue G-250 (w/v), 4.75% ethanol (w/v) and 8.5% O-phosphoric acid (w/v).

Procedure
Extraction of soluble protein
0.5 gm of chopped fresh leaf material was homogenized in 1.5 ml of 0.1 M phosphate buffer (extraction buffer) at / below 4°C with the help of a pre
cooled mortar and pestle; and kept in ice during the process of
homogenization. The homogenate was transferred to a 2 ml eppendorf and
centrifuged at 5000 rpm for 20 min at 4°C. An equal amount of chilled 10%
TCA was added to 0.5 ml of the supernatant, which was again centrifuged at
3300 rpm for 30 min. The supernatant was discarded and the pellet left was
washed with acetone. It was then dissolved in 1 ml of 0.1 N NaOH.

Estimation of total soluble protein
To 0.2 ml aliquot, 1 ml of Bradford’s reagent was added and vortexed. The
tubes were kept for 10 min for optimal color development. The absorbance
was then recorded at 595 nm on a Beckman spectrophotometer (DU 640,
Fullerton, USA). The soluble protein concentrations were quantified with the
help of a standard curve prepared from the standard of Bovine Albumin
Serum (BSA) from Sigma, USA. The protein content was expressed in mg g⁻¹
fw.

B. Soluble amino acids
Lee and Takahashi’s method (1966) was used for the estimation of soluble
amino acids.

Preparation of Reagents
i. Citrate buffer (0.5 M, pH 5.5)
0.5 M citric solution was prepared by dissolving 9.6060 gm citric acid in DDW
and final volume was made 100 ml. 0.5 M sodium citrate solution was prepared
by dissolving 14.7050 gm of sodium citrate in DDW and final volume was made
to 100 ml. Two components of the buffer were mixed in an appropriate amount
to maintain the pH of buffer 5.5.

ii. 55% glycerol
It was prepared by mixing 55 ml of glycerol and 45 ml of DDW.
Material and Methods

iii. 1.0% ninhydrin solution

Ninhydrin solution was prepared by dissolving 1.0 g of ninhydrin in 0.5 M citrate buffer to a final volume of 100 ml.

Procedure

0.5 g of fresh leaf material was ground in 5 ml of absolute ethanol with the help of mortar and pestle and transferred to the centrifuge tube. It was then centrifuged at 5000 rpm for 10 min. at 4°C. After that supernatant was taken in a test tube and alcohol was evaporated by incubating the tubes at 80°C for 1 hour in the water bath. Pellets were dissolved in 10 ml of 0.5 M citrate buffer (pH 5.5) using vortex vigorously. 0.5 ml of aliquot was taken from this and 1.5 ml of 55% Glycerol and 0.5 ml Ninhydrin solution was added to it. The vials were kept in water bath for 20 min at 100°C and observe the development of a purple blue colour. The volume in each vials were made upto 6 ml by adding citrate buffer. Absorbance was recorded at 570 nm on UV-vis Spectrophotometer (Model DU 640 B, Beckman, USA).

Calibration curve was prepared from Glycine (Sigma) of different concentrations to calculate the amino acid content in different samples. The concentration was expressed in mg g⁻¹ fr.wt.

C. Nitrate content

Nitrate content of leaves was estimated using the method given by Grover et al. (1978) method.

0.5 gm fresh leaves were taken in conical flask, 50 mg charcoal and 10 ml distilled water were added to it and boiled for 4-5 mins. After filtration, the volume was made upto 50 ml by adding DDW. 1 ml of this aliquot was taken and 0.5 ml CuSO₄ solution, 0.25 ml hydrazine sulfate, 0.25 ml of 0.1 N NaOH were added to it. The vials were kept in water bath incubator for 10 min's at 33°C and then transferred to ice and 0.5 ml chilled acetone and 1.0 ml sulphanilamide and NEDD [N(1-napthyl) ethylene diamine dihydrochloride] were added. Make the
Material and Methods

A volume of 6 ml by adding 1.5 ml DDW and the vials were kept for 20 min’s for colour development. Optical density was measured at 540 nm on Beckman DU 640 spectrophotometer. The nitrate content was expressed as n mole g⁻¹ fr.wt. The concentration of nitrate was determined against the standard curve prepared by using KNO₃ (potassium nitrate) solution.

Preparation of Reagents:
i. 0.1 N NaOH: Prepared by dissolving 0.4 gm NaOH in 100 ml DDW.
ii. 0.01 M Hydrazine sulfate: Prepared by dissolving 130.12 mg hydrazine sulfate in 100 ml DDW.
iii. 10% Acetone: Prepared by dissolving 10 ml acetone in 100 ml of DDW.
iv. 1% Sulphanilamide: Prepared by dissolving 1.0 gm sulphanilamide in 100 ml of 1 N HCl.
v. 0.02% N-1 Naphthyl ethylene diamine dihydrochloride (NEDD): Prepared by dissolving 0.02 gm NEDD in 100 ml DDW.
vi. Copper sulphate (CuSO₄)- Zinc sulphate (ZnSO₄) catalyst

Solution A: It was prepared by dissolving 31.36 mg of CuSO₄ in 100 ml of DDW.
Solution B: It was prepared by dissolving 10 ml of ZnSO₄ in 100 ml of DDW.
Final catalyst solution was prepared by mixing 10 ml of solution A in 100 ml of Solution B.

D. Nitrate reductase activity

Nitrate reductase activity was estimated by Klepper et al. (1971).

Preparation of Reagents
i. 0.4 M KNO₃: Prepared by dissolving 4.04 gm KNO₃ in 100 ml DDW.
ii. 1% Sulphanilamide: Prepared by dissolving 1 gm sulphanilamide in 100 ml of 1N HCl.
iii. 1 N-HCl: Prepared by dissolving 8.4 ml HCl in 91.6 ml DDW.
iv. 0.02% NEDD: Prepared by dissolving 0.02 gm NEDD in 100 ml DDW.
0.1 M phosphate buffer (pH 7.2):
The solution of sodium dihydrogen phosphate (NaH$_2$PO$_4$) and disodium hydrogen phosphate (Na$_2$HPO$_4$) was prepared in the following manner:

**Solution A**: 1.42 g of Na$_2$HPO$_4$ was dissolved in DDW and the volume was made to 100 ml.

**Solution B**: 0.78 g of NaH$_2$PO$_4$ was dissolved in DDW and the volume was made to 50 ml.

Solution A and B were mixed in an appropriate ratio to adjust the pH at 7.02

**Procedure**

0.5 gm fresh leaves with 3.0 ml phosphate buffer (pH=7.2) were kept in vials with 3.0 ml of KNO$_3$ (0.4 M). The vials were kept in vacuum dessicator and vacuum infiltration was done for 30 seconds interval till the leaves settle down. The vials were kept in water bath incubator for 1 hr at 33°C under dark condition. After incubation, the vials were kept in hot water for 5 mins to stop the reaction. 0.2 ml aliquot was taken and 1.0 ml of sulphanilamide and 1.0 ml of NEDD was added to it. The final volume was made upto 6.0 ml by DDW. The vials were kept in dark for 20 mins for colour development. Optical density was measured at 540 nm on Beckman DU 640 spectrophotometer. The corresponding concentration of nitrate was determined against the standard curve of nitrite prepared by NaN0$_2$ (Sodium nitrate) solution. The nitrate reductase activity was expressed as µ mole g$^{-1}$ fr. wt. hr$^{-1}$

**E. Soluble sugar content**
The soluble sugar was estimated by the method of Dey (1990).

**Preparation of reagent**

i. **5% phenol**

Phenol solution was prepared by mixing 5 g of phenol in 95 ml of DDW.

ii. **90% ethanol**

It was prepared by mixing 90 ml of pure ethanol and 10 ml of DDW.
Procedure

0.5 g fresh leaf materials was kept in 10 ml of alcohol for 1 hour at 60°C in incubator. The extract was then decanted into a 25 ml volumetric flask and the residue re-extracted. Final volume was made up to 25 ml by adding alcohol. 1 ml aliquot was transferred to a thick walled test tube and 1.0 ml of 5% phenol was added to it and mixed thoroughly, 5 ml of analytical grade sulphuric acid was then added to it and mixed thoroughly by vertical agitation with a glass rod. For exothermic reaction the test tube was cooled in the air. Absorbance was recorded at 485 nm on Beckman DU 640 spectrophotometer
The corresponding concentration was determined against a standard curve prepared by using a glucose solution. The amount of sugar was expressed as mg g⁻¹ fw⁻¹.

F. Proline content

The proline content was estimated by the method of Bates et al. (1973) as described below:

Preparation of reagents
i. 3% (w/v) Sulphosalicylic acid
3 g sulphosalicylic acid was dissolved in DDW to make a final volume of 100 ml.
ii. 6 N Orthophosphoric acid
   It was prepared by mixing 38.10 ml of orthophosphoric acid in 61.90 ml of DDW.
iii. Ninhydrin solution
    1.25 g of ninhydrin was added in 30 ml of glacial acetic acid and 20 ml of 6 N orthophosphoric acid, and agitated continuously till dissolved. The final volume was up to 100 ml with DDW.

Procedure

0.5 g of fresh leaf material was taken and homogenized with 10 ml of sulphosalicylic acid with the help of mortar and pestle. The homogenate was centrifuged at 5,000 rpm for 10 min. 2 ml supernatant was taken in a test tube to
which 2 ml of acid ninhydrin and 2 ml of glacial acetic acid were added. The mixture was boiled at 100°C in a water bath for 1 hr. The reaction was stopped by keeping the test tubes in an ice bath for 10 min. Then 4 ml of toluene was added to each tube and mixed vigorously on a cyclomixer for 10-15 secs in order to facilitate quick diffusion/movement of chromophores from the aqueous phase to non-aqueous phase. The toluene layer (upper) was separated from the mixture and absorbance was read at 520 nm on a spectrophotometer.

The corresponding concentration of proline was determined against the standard curve processed in the same manner using L-proline (Sigma). The amount of proline was expressed as mg g⁻¹ fw.

Experiment 5: Variation in lipid peroxidation, enzymatic and non-enzymatic system of *P. corylifolia* L, exposed to gamma rays.

The fresh leaves randomly harvested from middle portion of *P. corylifolia* L. plants at Preflowering, flowering and post flowering stages (45, 90, 145 DAS) to investigate variation in following biochemical aspects.

- Malondialdehyde (MDA)
- Superoxide dismutase (SOD)
- Catalase (CAT)
- Ascorbate peroxidase
- Glutathione reductase

**A. Malondialdehyde content**

Malondialdehyde content in leaves of *P. corylifolia* was determined by the method given by Heath and Packer (1968).
Material and Methods

Reagent preparation:

i. 1% trichloroacetic acid (TCA)

1g of TCA was dissolved in DDW and volume was made to 100 ml.

ii. 20% trichloroacetic acid (TCA)

20 g of TCA was dissolved in DDW and volume was made to 100 ml.

iii. 0.5% Thiobarbituric acid (TBA)

0.5g of TBA was dissolved in 100 ml of 20% TCA.

Procedure

0.05gm fresh tissue was ground in 2 ml of 1% TCA with a mortar and pestle. The ground material was centrifuged at 10,000 rpm for 10 mins. 1 ml of supernatant was taken in a separate test tube, to which 4.0 ml of 0.5% TBA was added. The mixture was heated at 95°C for 30 mins. It was then quickly cooled in an ice bath and centrifuged at 10000 rpm for 10 mins to clarify the reaction mixture. The absorbance of the supernatant at 532 nm was read and corrected for unspecific turbidity by subtracting the value at 600 nm on UV-vis spectrophotometer (Model DU 640 B, Beckman, USA). The concentration of MDA was calculated using an extinction coefficient of 155 mM⁻¹cm⁻¹ employed in the following formula:

\[
MDA \ (n\ mol\ g^{-1}\ fr.\ wt.) = \frac{(A_{532} - A_{600}) \times V \times 1000}{155 \times W}
\]
Where,

\[ A_{532} = \text{Absorbance at } 532 \text{ nm} \]

\[ A_{600} = \text{Absorbance at } 600 \text{ nm} \]

\[ V = \text{Extraction volume} \]

\[ W = \text{Fresh weight of tissue} \]

B. *Superoxide dismutase activity (SOD EC 1.15.1.1)*

The method given by Dhindsa et al. (1981) was followed with a slight modification for the estimation of SOD activity.

**Preparation of reagents**

i. **1 M Sodium bicarbonate solution (NaHCO₃)**
   It was prepared by dissolving 0.84 g of sodium carbonate in distilled water and the volume was made up to 100 ml.

ii. **200 mM Methionine solution**
   It was prepared by dissolving 2.98 g of methionine in DDW and the volume was made up to 100 ml.

iii. **2.25 mM Nitroblue tetrazolium (NBT) solution**
    NBT solution was prepared by dissolving 0.184 g of NBT in 100 ml of DDW.

iv. **3 mM EDTA**
   It was prepared by dissolving 0.088 g EDTA in 100 ml DDW.

v. **60 µM Riboflavin**
   It was prepared by dissolving 2.3 mg of the chemical in 100 ml of DDW.

vi. **Extraction buffer**
   It was prepared from 0.1 M phosphate buffer (pH = 7.5). The solution of monobasic potassium phosphate (KH₂PO₄) and dibasic potassium phosphate (K₂HPO₄) were first prepared in the following manner:

   **Solution A:** 0.68 g of KH₂PO₄ was dissolved in DDW and the volume was made up to 50 ml.
Material and Methods

Solution B: 1.74 g of K$_2$HPO$_4$ was dissolved in DDW and the volume was made up to 100 ml.
To prepare the extraction buffer, solution A and B were mixed in an appropriate ratio and pH was adjusted to 7.5. To 100 ml of this buffer, 1 g of polyvinyl pyrolidine (PVP), 1 ml of Triton x 100, and 3 mM EDTA were added.

vii. Reaction buffer

0.1 M phosphate buffer (pH 7.5) was used as extraction buffer. The solutions of potassium dihydrogen phosphate (KH$_2$PO$_4$) and dipotassium hydrogen phosphate (K$_2$HPO$_4$) were prepared in the following manner:

Solution A: 0.68 g of KH$_2$PO$_4$ was dissolved in DDW and the volume was made up to 50 ml.
Solution B: 1.74 g of K$_2$HPO$_4$ was dissolved in DDW and the volume was made up to 100 ml.

Solution A and B were mixed in an appropriate ratio to adjust the pH at 7.5. To 100 ml of this buffer 1.0 g PVP was added.

Procedure

0.05 g of fresh material was homogenized in 2.0 ml of extraction mixture with the help of mortar and pestle. The process was carried out under cold condition (4°C). The mortar and pestle was kept in ice during the course of homogenization. The homogenate was transferred to centrifuge tubes and centrifuged at 10,000 rpm for 10 min. at 4°C.

Enzyme assay

Superoxide dismutase (SOD) activity in the supernatant was assayed by its ability to inhibit the photochemical reduction. The assay mixture, consisting of 0.5 ml of enzyme extract, 1.5 ml reaction buffer, 0.2 ml of methionine, 0.1 ml NaHCO$_3$, 0.1 ml NBT solution, 0.1 ml EDTA, 0.1 ml riboflavin (immediately after keeping in light) tubes which were incubated under the light of 15 W
influorescent lamp for 10 min at 25/28°C. Blank A, containing all the above substances of the reaction mixture, along with the enzyme extract was placed in the dark. Blank B, containing all the above substances of reaction mixture except enzyme was placed in light along with the sample. The reaction was terminated by switching off the light and the tubes were covered with a black cloth. The non-irradiated reaction mixture containing enzyme extract did not develop light blue color. Absorbance of samples along with blank B was read at 560 nm against the blank A. The difference of % reduction in the color between blank B and the sample was then calculated.

50% reduction in the color was considered as one unit of enzyme activity and the activity was expressed in Enzyme Unit (EU) mg⁻¹ protein h⁻¹.

SOD activity (EU mg⁻¹ protein h⁻¹) =

\[
\frac{\text{% Reduction in color between blank and sample} \times \text{Dilution factor} \times 60}{50 \times \text{Incubation time} \times \text{mg protein in sample}}
\]

C. Catalase activity (CAT EC 1.11.36): Catalase (H₂O₂,H₂O₂ oxidoreductase; EC 1.11.11.6):

Activity in the leaves was determined by the method of Aebi (1984) after a slight modification.

Preparation of reagent

i. Extraction buffer

0.1 M phosphate buffer (pH 7.5) was the basic component of the extraction buffer. Solution A: 0.68 g of KH₂PO₄ was dissolved in DDW and the volume was made up to 50 ml.

Solution B: 1.74 g of K₂HPO₄ was dissolved in DDW and the volume was made up to 100 ml.
Solution A and B were mixed in an appropriate ratio and pH was adjusted to 7.3. To 100 ml of this buffer, 1.0 g PVP, 1.0 ml Triton x 100, and 0.11g of EDTA were added.

ii. Reaction buffer
It was prepared from 0.5 M phosphate buffer with pH 7.5. The two buffer components were mixed in such a way that pH was adjusted to 7.5 (measured by a pH meter).

Solution A: 3.49g of KH$_2$PO$_4$ was dissolved in DDW and the volume was made up to 50 ml.

Solution B: 8.70 g of K$_2$HPO$_4$ was dissolved in DDW and the volume was made up to 100 ml.

Solution A and B were mixed in an appropriate ratio and pH was adjusted to 7.5.

iii. 0.3% H$_2$O$_2$
0.3 ml of H$_2$O$_2$ was mixed with 9.7 ml of DDW.

iv. 3 mM EDTA
0.088 g was dissolved in DDW and the volume was up to 100 ml.

Procedure
0.05 g of the fresh tissue was homogenized in 2 ml of extraction mixture under cold condition. The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was used for quick assay.

Enzyme assay:
Catalase activity was determined by monitoring the disappearance of H$_2$O$_2$ by measuring a decrease in absorbance at 240 nm. Reaction was carried in a final volume of 1 ml of reaction mixture containing reaction buffer with 0.5 ml 3 mM EDTA, 0.2 ml of enzyme extract and 0.1 ml H$_2$O$_2$. The reaction was allowed to run for 5min. Activity was calculated by using Extinction Coefficient ($E$) 0.036 mM$^{-1}$ cm$^{-1}$ and expressed in Enzyme Units (mg$^{-1}$ protein). One unit of enzyme determines the amount necessary to decompose 1 μmol of H$_2$O$_2$ per min. at 25°C.
**Procedure**

1.0 g of the fresh material was ground in 4 ml of extraction buffer and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected and used for the assay immediately or kept under deep freeze conditions.

**D. Ascorbate peroxidase activity (APX EC 1.11.1.11)**

Ascorbate peroxidase (L-Ascorbate: H₂O₂ oxidoreductase; EC 1.11.1.11) activity was estimated by the method used by Nakano and Asada (1981).

**Preparation of reagent**

i. **Extraction buffer**

100 mM Phosphate buffer was prepared as:

Solution A: 1.36 g KH₂PO₄ was dissolved in DDW and the volume was made up to 100 ml.

Solution B: 1.74 g of K₂HPO₄ was dissolved in DDW and the volume was made up to 100 ml.

To prepare the extraction buffer, the two components A and B were mixed together and pH was adjusted to 7.0. To 100 ml of this buffer, 1.0 g PVP, 1.0 ml Triton X 100, and 0.11 g of EDTA was added.

ii. **Reaction buffer**

50 mM PO₄-buffer (pH 7.2/7.0) was prepared as follows:

Solution A: 50 mM NaH₂PO₄ was prepared by dissolving 1.142 g in DDW and volume was made up to 100 ml.

Solution B: 50 mM Na₂HPO₄ was prepared by dissolving 0.707 g of this chemical in DDW and volume was made up to 100 ml. 50 mM PO₄-buffer (pH 7.0/7.2) was prepared from these two A and B components by mixing them in an appropriate ratio and pH was adjusted to 7.4.

iii. **0.5 mM Ascorbate**

It was prepared by dissolving 44 mg of L-ascorbate in DDW and the volume was made up to 100 ml.
iv. 0.3% (v/v) H₂O₂
It was prepared by mixing 1 ml of 30% H₂O₂ with 99 ml of DDW.

Enzyme assay
Ascorbate peroxidase activity (APX) activity was determined by the decrease in absorbance of ascorbate at 290 nm due to its enzymatic breakdown. 1.0 ml of the reaction buffer contained 0.5 mM ascorbate, 0.1 mM H₂O₂, 0.1 mM EDTA and enzyme extract. The reaction was allowed to run for 3/5 min at 25°C. APX activity was calculated by using extinction coefficient (ε) 2.8 mM⁻¹ cm⁻¹ and expressed in Enzyme Units (EU) mg⁻¹ protein. One unit of enzyme determines the amount necessary to decompose 1 μmol of substrate consumed per min. at 25°C.

D. Glutathione reductase activity (GR EC 1.6.4.2)
Glutathione reductase (EC 1.6.4.2) activity was determined by the method of Foyer and Halliwell (1976) modified by Rao (1992).

Preparation of reagent
i. Extraction buffer
100 mM Phosphate buffer was prepared as:
Solution A: 1.36 g KH₂PO₄ was dissolved in DDW and the volume was made up to 100 ml.
Solution B: 1.74 g of K₂HPO₄ was dissolved in DDW and the volume was made up to 100 ml.
To prepare the extraction buffer, the two components A and B were mixed together in an appropriate amount and pH was adjusted to 7.0. To 100 ml of this buffer, 1.0 g of PVP, 1.0 ml Triton X 100, and 0.11 g of EDTA were added.

ii. Reaction buffer
0.25 M/0.1M Tris-buffer was prepared as follows:

iii. 0.2 mM NADPH
It was prepared by dissolving 2 mg of the chemical in 10 ml of DDW.
iv. 0.5 mM oxidised glutathione (GSSG)

It was prepared by dissolving 4 mg of the chemical in 13 ml of DDW.

Procedure

0.5 g of the fresh material was ground in 2 ml of extraction buffer and centrifuged at 10,000 g for 10 min. The supernatant was collected and used for the assay immediately or stored in deep freeze condition.

Enzyme assay

GR activity was determined by monitoring the glutathione-dependent oxidation of NADPH at its absorption maxima of the wavelength 340 nm. 1.0 ml reaction mixture contained 0.2 mM NADPH, 0.5 mM GSSG and 50 μl of enzyme extract.

The reaction was allowed to run for 5 min at 25°C. Corrections were made for any GSSG oxidation in the absence of NADPH. The activity was calculated by using Extinction Coefficient (ε) 6.2 mM$^{-1}$ cm$^{-1}$ and expressed in Enzyme Units mg$^{-1}$ protein. One unit of enzyme determines the amount necessary to decompose 1 μmol of NADPH per min at 25°C.

Experiment 6: Variation in non-enzymatic aspects (ascorbate-glutathione cycle) of *P. corylifolia* L. exposed to gamma rays.

The fresh leaves randomly harvested from middle portion of *Psoralea corylifolia* L. plants at Preflowering, flowering and post flowering stages (30, 60, 120) DAS to investigate variation in non-enzymatic aspects.

- Reduced ascorbate, oxidized ascorbate and total Ascorbate content

Ascorbate (Asc), dehydroascorbate (DAsc) and total ascorbate (Asc + DAsc) were estimated by a modified method of Law *et al.* (1983).

A. Reagent preparation

i. Extraction buffer (150 mM phosphate buffer, pH 7.4)

The solution of 150 mM monobasic potassium phosphate (KH$_2$PO$_4$) was prepared by dissolving 2.04 g of KH$_2$PO$_4$ in DDW and volume was made to 100
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ml. 150 mM dibasic potassium phosphate K$_2$HPO$_4$ solution was prepared by dissolving 2.61 g of the K$_2$HPO$_4$ in DDW and volume was made to 100 ml. Two components of buffer were mixed in an appropriate amount to maintain the pH of buffer 7.4 that was monitored with the pH meter.

ii. 5M sodium hydroxide

It was prepared by dissolving 2g of NaOH in 10 ml of DDW

iii. Reaction buffer (150 mM sodium phosphate buffer, pH 7.4)

150 mM NaH$_2$PO$_4$ was prepared by dissolving 1.17 g of NaH$_2$PO$_4$ in DDW and volume was made to 50 ml. 150 mM Na$_2$HPO$_4$ was prepared by dissolving its 1.06 g of Na$_2$HPO$_4$ in DDW and volume was made to 50 ml. two components of buffer were mixed in an appropriate amount to maintain the pH of buffer 7.4 that was monitored with the pH meter.

iv. 2 mM Magnesium chloride (MgCl$_2$)

It was prepared by dissolving 0.40 g of MgCl$_2$ in 100 ml of 0.1 M-Tris buffer (pH 8.0)

v. 10 mM Dithiothrieyotol (DDT)

It was prepared by dissolving 7.7mg of DDT in 5 ml of DDW

vi. 10% Trichloroacetic acid (TCA)

It was prepared by dissolving 10 g of TCA in 100 ml of DDW/

vii. 44% (v/v) O-Phosphoric acid

It was prepared by mixing 44 ml of H$_3$PO$_4$ in DDW and volume was made to 100 ml with DDW

viii. 70% Ethanol

It was prepared by mixing 70 ml of ethanol in DDW to a volume of 100 ml DDW.

ix. 4% bipyridyl

It was prepared by dissolving 2g of bipyridyl in 50 ml of 70% ethanol

x. 3% (w/v) Ferric chloride (FeCl$_3$)
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It was prepared by dissolving 0.75 g of FeCl₃ in DDW and volume was made to 25 ml with DDW.

Procedure

0.5 g of fresh leaf was ground in 2 ml of extraction buffer and centrifuged at 10,000 rpm for 10 min. The supernatant was distributed in two separate microcentrifuge tubes (400 μl in each) for the assay of total ascorbate (As + DAs) and reduced ascorbate. DAs concentration was then deduced from the difference. To each tube 200 μl of 10% TCA was added and vortexed mixed. 10 μl of NaOH solution was then added to it, mixed and the mixture was centrifuged for 2 min in microcentrifuge. To 200 μl of the supernatant, 200 μl of 150 mM of NaH₂PO₄ and 200 μl of water were added. To another 200 μl of supernatant, 200 μl of buffer and 100 μl of 10 μl of 10 mM DDT were added and thoroughly mixed. Then 100 μl of 0.5% N-Ethylmaleimide was added to each tube. Both samples were vortexed mixed and incubated at room temperature for 30 min. To each tube was then added 400 μl of 10% TCA, 400 μl of 44% H₃PO₄, 4 l of 4% bipyridyl and 200 l of 3% FeCl₃. After vortexed mixing, samples were incubated at 33°C for 60 min. and the absorbance was recorded at 525 nm on uv-vis spectrophotometer (Model Du 640, Beckman, USA)

Calibration curve was prepared from different concentration of ascorbic acid (Sigma). The amount was expressed in n mol g⁻¹ fr. wt.

Reduced Glutathione, oxidized Glutathione and total Glutathione

Reduced Glutathione, oxidized Glutathione and total Glutathione contents were determined by the GSSG recycling method of Anderson (1985).

Reagent Preparation

i. 5% sulphosalicylic acid

5.0 g of sulphosalicylic acid was dissolved in 100 ml of DDW.
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ii. Reaction buffer (0.1 M phosphate buffer, pH 7.0)

The solution of 0.1 M monobasic potassium phosphate (KH$_2$PO$_4$) was prepared by dissolving 1.36 g of KH$_2$PO$_4$ in DDW and volume was made to 100 ml. 0.1 M dibasic potassium phosphate (K$_2$HPO$_4$) solution was prepared by dissolving 1.74 g of K$_2$HPO$_4$ in DDW and volume was made to 100 ml. Two components of the buffer were mixed in an appropriate amount to maintain the pH of buffer 7.8 that was monitored with the pH meter. To 100 ml of this buffer 0.11 mg of EDTA was added.

iii. 0.5% Sodium bicarbonate (NaHCO$_3$)

It was prepared by dissolving 0.5 g of NaHCO$_3$ in DDW and final volume was made to 100 ml.

iv. 0.4% Nicotinamide adenine dinucleotide phosphate reduced (NADPH)

It was prepared by dissolving 20 mg of NADPH in 5 ml of 0.5% NaHCO$_3$

v. 0.15% Dithionitro benzoic acid (DTNB)

It was prepared by dissolving 6 mg of DTNB in 4 ml of 0.5% NaHCO$_3$

Procedure

0.5 g of fresh leaf was homogenized in 2 ml of 5% sulphosalicylic acid under cold condition. The homogenate was centrifuged at 10,000 rpm for 10 min. 0.5 ml of aliquot was taken in a micro centrifuge tube, to which 0.6 ml of reaction buffer and 40 µl of DTNB was added. Absorbance for determination of GSH was read at 412 nm on uv-vis spectrophotometer (Model DU 640, Beckman, USA) after 2 min. To the same tube 50 µl of NADPH and 2 µl of GR was added for the determination of total glutathione. Substracting the reduced glutathione from
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total glutathione determines the oxidized glutathione. The reaction was allowed
to run for 30 min. at 25°C. The change in absorbance at 412 nm on UV-VIS
spectrophotometer (Model DU 640, Beckman, USA) was recorded. Values are
corrected for the absorbance of supernatant and DTNB. Calibration curve was
prepared from reduced glutathione (Sigma) of different concentration to
calculate the GSH, GSSH and (GSH + GSSH) content in samples, treated similar
to that of aliquots, described as above. The amount of GSH, GSSH and (GSH +
GSSH) was expressed as n mol g\(^{-1}\) fr. wt.

Seed yield
At harvest stage, ten random plants from each treatment were used to record the
following data: number of seeds per plant and seed index (weight of 100 seeds).

Experiment 7: Alteration in protein profile of *Psoralea* seeds exposed to different
doses of gamma rays

Protein profile:
Protein profile was studied using SDS-PAGE by Laemmli (1970) method.

Procedure
0.5 g of the fresh plant tissue was homogenized in 2 ml of extraction buffer in
pre-cooled mortar and pestle in ice. The homogenate was centrifuged at 10,000
rpm for 10 minutes at 4°C. Supernatant obtained was divided in two parts. One
part was used for the estimation of protein (by Bradford’s method) and other
part was used for gel loading. After knowing the concentration of the protein in
the sample, the remaining supernatant was treated with 10% TCA and
centrifuged at 5000 rpm. The supernatant was discarded and pellet was washed
with chilled acetone and dissolved in desired amount of sample buffer and
sample dye (3:1). It was then heated for 2-3 minutes at 100°C.
Molecular marker

A small amount of commercially available molecular markers mixture, covering a wide range of molecular weights (3 -205 KDa and 26 -180 KDa) was used.

Gel solution preparation A 12% resolving gel and 4% stacking gel was prepared as follows:

<table>
<thead>
<tr>
<th>COMPOSITION</th>
<th>RESOLVING GEL (mL)</th>
<th>STACKING GEL (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O</td>
<td>9.90</td>
<td>6.80</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>12.00</td>
<td>1.70</td>
</tr>
<tr>
<td>1.5M Tris (pH 8.8)</td>
<td>7.50</td>
<td>-</td>
</tr>
<tr>
<td>1.0M Tris (pH6)</td>
<td>-</td>
<td>1.25</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.40</td>
<td>0.10</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.40</td>
<td>0.10</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.016</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Gel casting and gel loading

Gel solutions were poured in the gel slab. After its polymerization, equal amount of protein of each sample was loaded in the wells. Marker was also loaded in one of the wells of the same gel. The proteins were allowed to run in the gel for 10 hrs with current 40 m amp. with bromophenol blue as the tracking dye. When the dye reaches the base of the gel, current was terminated. Gel was separated from the slab and poured in staining solution for 8 hrs. After that gel was put into destaining solution. The destained solution was changed after every 30 minutes till the background of the gel became clear and protein bands were visible. Molecular weights of the proteins were determined with reference to protein markers of known molecular weights using Gel documentation system from Uvitec Cambridge U.K., with software UV photo V. 10.
Preparation of Reagents

i. Tris buffer (1.5 M, pH 8.8)
It was prepared by dissolving 18.04 g of Tris base in 70 ml of DDW, pH was
adjusted to 8.8 with 5 N-HCl and final volume was made to 100 ml.

ii. Tris buffer (1.0 M, pH 6.8)
It was prepared by dissolving 12.00 g of Tris base in 70 ml of DDW, pH was
adjusted to 6.8 with 5 N-HCl and final volume was made to 100 ml.

iii. 5 N-Hydrochloric acid (HCl)
It was prepared by mixing 43.3 ml of HCl with 56.7 ml DDW.

iv. Sample preparation buffer
It was prepared from 2.5 ml of 1.0 M Tris HCl (pH 6.8), 1 g of SDS and 2.5 ml of
β-mercaptoethanol were added to it. Final volume was made to 40 ml.

v. 10% Trichloroacetic acid (TCA)
10 g of TCA was dissolved in DDW to a final volume of 100 ml.

vi. 0.1 N-Sodium hydroxide (NaOH)
0.4 g of NaOH was dissolved in 100 ml of DDW.

vii. Bradford’s reagent
It was prepared as mentioned earlier;

viii. 30% Acrylamide and bis-acrylamide
It was prepared by dissolving 14.55 g of acrylamide and 0.45 g of bisacrylamide
in DDW and volume was made to 50 ml. The solution was then stored in the
dark bottle and kept at 4°C.

ix. 10% Ammonium per sulphate (APS)
It was prepared by dissolving 0.1 g of APS in DDW to a final volume of 1 ml.

x. 10% Sodium dodecyl sulphate (SDS)
It was prepared by dissolving 0.1 g SDS in DDW to a final volume of 1 ml.

xi. Sample dye
It was prepared by dissolving 5 mg bromophenol blue in 5.8 ml glycerol and
volume was made to 10 ml by adding DDW.
xii. Electrode buffer
It was prepared by dissolving 3 g tris base, 14.42 g glycine and 1 g SDS in DDW and volume was made to 1000 ml.

xiii. Gel staining solution
It was prepared by dissolving 1 g of Coomassie brilliant blue R to a mixture of 180 ml methanol, 180 ml DDW and 40 ml of acetic acid.

xiv. Gel destaining solution
It was prepared by mixing 100 ml glacial acetic acid, 400 ml methanol and 400 ml of DDW. The mixture was stirred well before use on a magnetic stirrer.

Experiment 8. Extraction and isolation of psoralen at different developmental stages by Thin layer Chromatography (TLC):
Leaves, roots and seeds from control as well as irradiated plants collected at different developmental stages of *Psoralen* were investigated following the method of Innocenti et al. (1977). Harvested biomass was dried in an oven at 55 °C for 16 h and powdered by Wiley Mill (Model No. 4276, Thomas Scientific). Dried powder was extracted with chloroform in Soxhlet apparatus for 48 h, and chloroform extract was concentrated. This extract contained furanocoumarins in plants in free form. The extract marc was refluxed for 2 h in 30 ml of 5% HCl. After cooling and filtration, an acid residue and an aqueous-acid phase were obtained. The acid residue was extracted with ethanol for 48 h and ethanolic extract was then concentrated (A). The aqueous-acid phase was extracted with ethanol after distillation of the solvent; the residue was dissolved in ethanol (B). Ethanolic solutions A and B were combined and vortexed for 3 min. and transferred to polypropylene micro-centrifuge (Eppendorf) tubes and centrifuged at 12000 x g for 10 min. The supernatant was loaded on an analytical silica gel plate (Silica -60G) and run in chloroform (the developing solvent) to isolate psoralen (Rf=0.40). The areas on TLC plate corresponding to psoralen were eluted separately for different plant parts with ethanol. Psoralen was then
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detected by HP 1100 DAD detector (Agilent technologies, Palo Alto, CA, USA) at 248nm. Quantification was obtained by corresponding standard used for calibration curve preparation.

experiment 9. isolation of psoralen and isopsoralen in Psoralea seeds by high performance liquid chromatography (HPLC) method.

Isolation of psoralen and isopsoralen was carried out following the method given by Zhao et al. (2005).

Instrumentation and chromatographic condition.

Reagents and Materials: The sample of P. corylifolia seeds were harvested at end of season (Oct-Nov) for analysis. Acetonitrile and water were of HPLC grade. Acetic acid was of analytical grade.

Standards of psoralen and isopsoralen were provided by Sigma Chemicals, Mumbai, India. Their purity is higher than 98.0%.

Apparatus and Column

The HPLC system consisted of binary pumps (Shimadzu LC-10AD, Japan), a UV detector (Shimadzu LC-10A, Japan), and a model 7725 I manual injector valve with a 20m l sample loop. The signals from the detector were analyzed with a computer equipped with a software of N2000 system (Hamdard University, India). An Alltech C18, 250-4.6 mm, 5m m, column (Alltima, China) was used for all chromatographic separations. The temperature of the column was kept at room temperature.

Chromatographic Conditions

The mobile phase was acetonitrile (solvent A) and 0.1% acetic acid–water (solvent B) in the gradient mode: 0—20 min, 60—50% B; 20—35 min, 50—40% B; 35—45 min, 40—30% B; 45—55 min, 30—20% B; 60 min, stop. The flow-rate was 1.0ml min⁻¹. The effluent was monitored at 245 nm.

Sample preparation

0.2 g sample of the fine-grinded powder was accurately weighted and extracted with 20 ml of 70% ethanol in ultrasonic bath for 30 min at 30°C and filtered. Five
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millilitres of the continual filtrate was diluted to 25 ml volumetric flask by the same solvent as sample solution and filtered through a 0.45-μm filter membrane before analysis. Twenty microliters of the sample solution was injected to HPLC column and separated under above chromatographic conditions.


Isolation Procedures

The essential oil of seeds (200g) of *P. corylifolia* was isolated separately by hydrodistillation (2 h) using a Clevenger type apparatus. The essential oils were dissolved in Et₂O, dried over anhydrous MgSO₄, filtered and the solvent was removed by evaporation on a water bath. The plant material was filtered off and the aqueous residues were extracted, first with *n*-hexane (250 ml) and then with Et₂O (250 ml), to remove traces of volatile compounds. The organic solvents, dissolved in the aqueous phase, were evaporated under reduced pressure at room temperature. The remaining aqueous extracts were treated with β-glucosidase (41 mg) from almonds (BioChemika, 1 g, Lot. 49290, Fluka) at 28 °C for 16 h and the volatile compounds were collected in *n*-hexane (0.5 ml) by hydrodistillation. All the essential oils and the volatile fractions were analysed by GC and GC-MS.

Gas Chromatography

GC analyses were accomplished with a HP-5890 Series II instrument equipped with HP-WAX and HP-capillary columns (30 m × 0.25 mm, 0.25 μm film thickness), working with the following temperature program: 60 °C for 10 min, rising at 5 °C/min to 220 °C; injector and detector temperatures, 250 °C; carrier gas, nitrogen (2 ml/min); detector, dual FID; split ratio, 1:30; injection, 0.5 ml. The identification of the components was performed, for both the columns, by the comparison of their retention time with those of pure authentic samples and by means of their linear retention indices (LRI) relative to the series of *n*-hydrocarbons. The relative proportions of the essential oil constituent
percentages were obtained by FID peak-area normalization, all relative response factors being taken as one.

**Gas Chromatography-Mass Spectrometry**

GC-MS analyses were performed with a Varian CP-3800 gas chromatograph equipped with a DB-5 capillary column (30 m x 0.25 mm; coating thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions: injector and transfer line temperatures, 220 °C and 240 °C, respectively; oven temperature programmed from 60 °C to 240 °C at 30 °C/min; carrier gas, helium at 1 ml/min; injection, 0.2 μl (10% hexane solution); split ratio, 1:30. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their linear retention indices relative to the series of n-hydrocarbons, and on computer matching against commercial (NIST 98 and ADAMS) besides home-made library mass spectra built up from pure substances and components of known oils and MS literature data. Moreover, the molecular weights of all the identified substances were confirmed by GC-CIMS, using MeOH as Cl ionizing gas.

**Solid Phase Particle Micro extraction (SPME Analysis)**

SPME analysis were performed with Supelco SPME devices, coated with polydimethylsiloxane (PDMS, 100μm), in order to sample the headspace of a portion (1g) of seed sample. Each plant aliquot was inserted into a 100ml glass conical flask and allowed to equilibrate for 20 min. After the equilibration time, the fibre was exposed to the headspace for 15 min at room temperature, and when the sampling was finished (5 min), the fibre was withdrawn into needle and transferred to injection port of GC and GC-MS system, operating in the same condition as described above for both the identification and quantification of the constituents, apart from the splitless injection mode and the injector temperature (250°C).
Statistical Analysis:
Each experiment was performed three times and all the determinations were obtained from ten replicates (N=10). Results are expressed as mean ± standard error. The data values were submitted for analysis of variance for each factor (dose and developmental stages) and their interaction. We used One-way ANOVA analysis (P≤ 0.05) DMRT test (Graph Pad Prism 5, 2003 Analytical Software).