Chapter II

MATERIALS AND METHODS

Selection of sites in the campus Pune University

Through the frequent and random visits to the entire campus of Pune university in different seasons, for suitability and convenience of this study, was divided into four different sites, which were representing the varied vegetation and soil types. The four selected sites are representing the ecological and environmental conditions prevailing in the entire campus. The study sites were named as I, II, III and IV. Each site was having an approximate area of 40 hectares. The exact locations of selected sites are shown in Plates V and VI.

Phytosociological studies

To investigate the allelopathic potential of different native and invaded weed species, phytosociological studies were conducted randomly at about 20 different spots in each selected site in Pune University campus, by employing the list count quadrat method of Misra (1968). The phytosociological investigations were carried out during January 2005 to December 2007 through weekly visits to each site when the vegetation was in full growth. From the twenty randomly selected quadrats at each site, the frequency, distribution, density and abundance were recorded for the observed native and invasive weeds.

The floristic composition, distribution and phytosociological associations were recorded by taking physical count of each plant species occurring in a quadrat, measuring 1×1m each (Plate VII). The different types of weed-weed (native-native, native-invasive, invasive-invasive) interactions and weed diversity were also recorded at each selected site (Plate VIII). The taxonomic identification was done with the help of Flora of Bombay Presidency (Cooke 1958) and Flora of Maharashtra (Singh et al. 2000) and herbaria were prepared by following standard methods. The specimens were also compared with the authentic herbaria of BSI, Western circle, Pune (M.S.) for confirming the identification.
Survey of Pune University Campus area with NAVigation System with Time and Ranging Global Positioning System analysis

The phytosociological studies of different weeds were supported by GPS mapping. For studying the distribution of native and invasive weed species in Pune University campus, GPS mapping method was used and the handset was ‘Garmin etrex’ make. The software used was Arc GIS 9 version 9.2 with which the map of Pune University campus was established (Plate IX).

Procedure for using GPS handset to record the coordinates

1. Start the GPS unit using Power button
2. GPS will automatically track the satellites
3. See the number of satellites (should be >4), also see the strength of the satellites using the bar graph
4. See the accuracy in the same page in meters (should be <7)
5. Press ‘page’ button 4 times to go the ‘Menu’ page
6. Select ‘Mark’ option and press Enter button
7. To save the waypoint press ‘Enter’ to answer ‘OK’
8. After saving waypoint, go to ‘Waypoints’ option using arrow buttons on the left side
9. Press Enter to select the latest waypoint down the list and Enter
10. Write the waypoint number in the waypoint column provided in the table
11. See the elevation (m) write the same in Z column
12. The latitude(N) should be written in Y column (degree decimals)
13. The longitude (E) should be written in X column (degree decimals)
14. Write the description of the waypoint (road, name of building etc.) in the remark column
The waypoints (200) located in the entire campus of Pune University were mainly selected to identify the geographical positions of the selected weeds *Cassia uniflora* and *Synedrella nodiflora*. Along with them other weed species were also located for reference. The waypoints were located at – Main gate area, Chhatrapati Shivaji Maharaj statue garden, jogging track cross road, behind geography and geology departments, botany dept., in front of Jaykar library, energy park area, near Aniket Canteen, international hostel area, area nearby hostel No. 6, main playground area near SET guest house, department of Astrology and astrophysics, VC Residence area, area nearby Pune university post office, area of General B.C. Joshi gate, in front of Ambedkar Bhavan, area nearby department of defense studies, area near department of geoinformatics and CINS, areas near by all staff quarters and hostels, departments of microbiology, management studies, health science, and entire range hill area.

**Selection of weed species**

Through the phytosociological studies the invasive weeds like *Cassia uniflora* Mill.non Spreng, *Synedrella nodiflora* (L) Gaertn., *Alternanthera tenella* Colla., *Euphorbia geniculata* Orteg., *Boerhaavia erecta* L., *Blainvillea acmella* L. and *Bidens biternata* Lour. were recorded and amongst these *Cassia uniflora* Mill. non Spreng. (family- Caesalpiniaceae) and *Synedrella nodiflora* (L.) Gaertn. (family- Asteraceae), were selected for present investigation, because of their dominance. The native weeds *Achyranthes aspera* L., *Acalypha ciliata* Forsk., *Triumfetta rhomboidea* Jacq., *Cassia absus* L., *Cassia obtusifolia* L., *Rauwolfia tetraphylla* L., *Oplismenus composites* P.Beauv. were selected for comparative studies along with the invasive weeds.

**Morphological characters and reproductive capacity**

The morphological characters like plant height, root length, number of branches, biomass (fresh and dry), biomass per quadrat and dry matter accumulation rate (DMAR) were recorded at flowering stage. Reproductive capacity of each weed was calculated by using the method of Salisbury (1942).

**Collection of leaf samples for physiological analyses**

The analyses of photosynthetic pigments, primary metabolites like reducing and total sugars, starch and proteins are important precursors for synthesis of many secondary metabolites and allelochemicals. These have also importance in growth, development of plants, flowering and fruiting, hence such analyses were attempted for
the selected invasive and native weeds as well as leachates, extracts and residues treated test crops. These weeds are usually acclimatized to different abiotic stress conditions; hence for understanding the stress tolerance mechanisms adapted by them, the antioxidants or osmoprotectants like proline, glycine betaine and lipid peroxidation were determined along with assay of different antioxidant enzymes, which have key role in scavenging the ROS.

The fresh leaf samples (third leaf from top) were randomly collected at full maturity stage from 25 different plants of native and invasive weeds from each site, which were brought to the laboratory in air tight plastic bags. These were cleaned and used for analysis of chlorophyll pigments, total sugars, starch, proteins, phenols and different antioxidant enzymes. For analysis of proline and glycine betaine, the leaf samples were dried under shade for four to five days in the laboratory at room temperature (25 ± 2°C) and then were used.

Physiological, biochemical and enzymological analyses of selected invasive and native weeds

As claimed by Reigosa et al. (1999), the multidisciplinary ecophysiological and molecular approaches are needed for understanding allelopathy in connection with the mechanism of action and adverse or positive effect of donor plant on recipient plant. Considering this the physiological, biochemical and enzymological analyses were attempted in selected invasive and native weed species and leachates, extracts and residues treated test plants.

Photosynthetic rate

Photosynthetic rate, stomatal conductance, transpiration rate, leaf temperature, vapour pressure deficit and water use efficiency (WUE) were recorded in field (in situ) for all the dominant weeds at different pre decided sites in the campus at the flowering stage. The weeds were selected randomly and the readings were recorded with Infra Red Gas Analyzer (IRGA – LA - 6400 system) by taking the third leaf from top in leaf chamber (Plate Xa). The average values of three such readings were recorded in table. Effect of CO₂ concentration was determined by gradual increase from 50 to 2000 µmol mol⁻¹. The temperature control is a standard feature of the LI-6400 system. It does not require external power supply or accessories. Photosynthetically active radiations were measured in the chamber near the leaf plane using a miniature sensor.
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Photosynthetic pigments

Photosynthetic pigments like chlorophyll a, chlorophyll b and total chlorophylls were extracted and estimated by using the method of Arnon (1949). The fresh leaf samples of selected invasive and native weed species were cut into small pieces and 1 g of leaf material was separately homogenized in 80% acetone in dark (to avoid photo oxidation), using mortar and pestle. The acetone extract was filtered through Whatman No. 1 filter paper and the final volume was made to 100 ml with 80% acetone. The absorbance of acetone extract was recorded at 645 and 663 nm using UV-visible spectrophotometer (Shimadzu-1601).

Total sugars

Total sugars were estimated by the method of Hodge and Hofreiter (1962). 1 g leaf samples (dried under shade) were homogenized separately in ten ml of 80% ethanol. This was condensed on hot water bath to approximately one ml and centrifuged at 5000 rpm for 15 minutes. The volume of the supernatant was adjusted to ten ml with DW. This supernatant (0.2 ml) was used for estimation of reducing sugars by using Dinitrosalicylic acid (DNSA) reagent. The red colour developed in reaction mixture was read at 510 nm on UV-visible spectrophotometer (Shimadzu-1601). D-glucose at the concentration of 100 µg ml⁻¹ was used to prepare the standard curve.

For estimation of total sugars five ml of 2.5 N HCl was added to five ml of above extracts of leaf samples and subjected to digestion on hot water bath. The acid digested samples were cooled and neutralized by adding anhydrous sodium carbonate. About five ml-distilled water was added in each neutralized sample and it was again centrifuged at 5000 rpm for 15 min. The final volume of supernatants was adjusted to 20 ml with distilled water and the same was used for estimation of total sugars. For estimation of total sugars, 0.2 ml extract was used. The blue colour developed in reaction mixture with Anthrone reagent was recorded at 630 nm on UV-visible spectrophotometer (Shimadzu-1601). D-glucose at the concentration of 100 µg ml⁻¹ was used to prepare the standard curve.

Starch

Starch content was estimated from 1 g of leaf samples (shade dried) by using Anthrone reagent as per the method described by Thayumanavan and Sadasivam (1984). The residues obtained during the extraction of sugars were re-suspended in digestion
mixture (6.5 ml of 52 % perchloric acid + 5 ml distilled water) and subjected to
digestion in cold condition at 0 °C in refrigerator for 20 minutes. The acid digested
samples were centrifuged at 5000 rpm for 15 min. The final volume of supernatants
was made to 100 ml with distilled water. This served as the source of starch; 0.2 ml of
this was used for estimation of starch. The olive green colour developed with
Anthrone reagent was recorded at 630 nm on UV-visible spectrophotometer
(Shimadzu-1601). The standard curve was prepared as described previously.

**Proteins**

Protein estimation was done according to Lowry et al. (1951) method. 1g of
leaf, samples (shade dried) were homogenized separately in 2.5 ml of 0.1M phosphate
buffer (pH 7.0). The extracts were centrifuged at 5000 rpm for 15 minutes at 4°C. The
supernatants were transferred to a sorvell tubes containing a mixture of 20 ml acetone
and 14 μl β- mercaptoethanol for precipitation of protein. The sample tubes were
stored at 0 °C for five hours and then centrifuged at 10,000 rpm for 20 minutes. The
supernatants were discarded and the pellets were dissolved in 2.5 ml of 1.0 N sodium
hydroxide solution. This was used as the source of protein. From this 0.2 ml, aliquot
was used to prepare the reaction mixture. The blue colour developed due to addition
of folin-phenol reagent was read at 660 nm on UV-visible spectrophotometer
(Shimadzu-1601). The BSA (Bovine Serum Albumin-fraction V) was used at the
concentration of one mg ml⁻¹ as standard protein, to prepare the standard curve.

**Phenols**

Total phenolics in the leaf samples were estimated as per the method of Farkas
and Kiraly (1962). 1g of leaf samples (shade dried) were homogenized separately in
ten ml of 80 % alcohol. The extracts were condensed on hot water bath to
approximately one ml and centrifuged at 5000 rpm for 15 min, volume of the
supernatant was adjusted to ten ml with distilled water. From the supernatant 0.2 ml
aliquot was used for estimation and the blue colour developed in reaction mixture
after addition of folin-phenol reagent was read at 650 nm on UV-visible
spectrophotometer (Shimadzu-1601). Tannic acid at the concentration of 100 μg ml⁻¹
was used to prepare the standard curve.

**Proline**

Proline content was estimated by using Bates et al. (1973) method. 1g of leaf
samples (shade dried) were homogenized separately in ten ml of three per cent
aqueous sulphasalicylic acid and then homogenate was filtered through Whatman No.1 filter paper. The filtrate (two ml) was taken in a test tube and to this two ml of glacial acetic acid and two ml of acid ninhydrin was added. The reaction mixture was kept in boiling water bath for one hour, after that the reaction was terminated by placing the tubes in ice bath. To this four ml of toluene was added and it was vigorously shaken for 20-30 seconds. After some time the upper toluene layer was separated and kept at room temperature. The red colour developed was measured at 520 nm on UV-visible spectrophotometer (Shimadzu-1601). Standard proline at the conc. of 100-µg ml⁻¹ was used to prepare the standard curve.

**Estimation of total free amino acids**

Total free amino acids were estimated as per the method described by Moore and Stein (1948). The fresh leaf samples were separately cut into small pieces and 0.5 g tissues were homogenized in ten ml (80%) alcohol. The extraction was repeated twice by five ml (80%) alcohol and the residues as well as the supernatants were pooled together. The extract was condensed on hot water bath to approximately one ml, centrifuged at 5000 rpm for 15 min. Volume of the supernatant was adjusted to ten ml with distilled water. From the supernatant 0.1 ml, aliquot was taken to which one ml of ninhydrin was added. Final volume was made to two ml with distilled water. The tube was heated in a boiling water bath for 20 min and five ml of the n-Propanol was added and mixed thoroughly. After 15 min., the intensity of the purple colour was recorded against the reagent blank in UV-visible spectrophotometer (Shimadzu-1601) at 570 nm. The reagent blank was prepared by taking 0.1 ml of 80% ethanol. Leucine at the concentration of 100 µg ml⁻¹ was used to prepare the standard curve.

**Estimation of glycine betaine**

Glycine betaine was estimated by using Ishitani (1993) method. For this 100 mg leaf, material was incubated in 20 ml of 1 N H₂SO₄ for 18 hrs. at 25 °C and the suspension was centrifuged at 2000 rpm for ten min. The reaction mixture of 0.25 ml supernatant and 0.75 ml 1 N H₂SO₄ were reacted with cold I₂KI reagent in a test tube. The well-mixed content of the tubes were cooled to 0 °C by keeping them in ice bath for two hours. These tubes were centrifuged again. Ten ml of ethylene dichloride was added to the precipitate in each tube and the absorbance was recorded at 365 nm on
UV-visible spectrophotometer (Shimadzu-1601). Glycine betaine concentration was calculated from calibration curve of 100 µg ml\(^{-1}\) betaine (Sigma make).

**Lipid peroxidation**

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) contents (Heath and Packer 1968). Fresh leaf samples (500 mg) were homogenized in 10 ml of 0.1% trichloro-acetic acid (TCA). The homogenate was centrifuged at 15,000 g for 5 min. To 2 ml of aliquot of the supernatant, 4 ml of 0.5% thiobarbituric acid (TBA) in 20% TCA was added. The mixture was heated at 95\(^\circ\)C for 30 min and then quickly cooled in ice bath. This was followed by centrifugation at 10,000 g for 10 min to remove suspended turbidity and then the absorbance was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted and the MDA content was calculated using its absorption coefficient of 155 mmol\(^{-1}\) cm\(^{-1}\).

**Relative water content**

The fresh weight and dry weight of leaves (3\(^{rd}\) leaf from top) of weeds were recorded to obtain relative water content. The dry weight was observed by heating the samples in a hot air oven at 60\(^\circ\)C for 24 h. This was done according to the method of Vinaykumar et al. (2007).

**Membrane stability index**

Membrane stability index (MSI) of weeds was calculated by following the method described by Sairam et al. (1997/98). 100mg fresh leaf material was cut into small pieces and washed with distilled water. These pieces then were added to 10ml distilled water in a test tube. The test tube was then kept in water bath adjusted at 40\(^\circ\)C for half an hour. After that the first EC (Electrical conductivity) was measured (EC I). The same mixture in the test tube was then kept in boiling water bath for 20 – 30 minutes. The test tube was cooled and second EC was measured (EC II). From these values MSI was determined.

**Assay of antioxidant enzymes**

**Peroxidase** [EC 1.11.1.7]

The enzyme assay was carried out by using Vidyasekharan and Durairaj (1973) method. 1g fresh leaf tissues were homogenized separately in five ml of 0.1 M phosphate buffer (pH 7.0). The extracts were centrifuged in refrigerated centrifuge (RC) at 15000 rpm for 20 minutes and the supernatants were used as enzyme source.
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The assay mixture of three ml contained 1.8 ml of 0.1 M phosphate buffer (pH 7.0), one ml freshly prepared 10 mM Guaiicol, 0.1 ml enzyme extract and 0.1 ml of 12.3 mM \( \text{H}_2\text{O}_2 \). Initial optical density was read at 430 nm and then increase in optical density was noted at the intervals of 30 seconds on UV-visible spectrophotometer (Shimadzu-1601). The amount of protein in 0.1ml of enzyme extract was calculated and the enzyme activity was expressed as \( \Delta \text{OD min}^{-1} \text{ mg}^{-1} \text{ protein} \).

**Polyphenol oxidase** [EC 1.14.18.1]

1g fresh leaf tissues were homogenized in chilled mortar in five ml of 0.1M phosphate buffer (pH 6.0). The extracts were centrifuged in RC at 15000 rpm for 20 minutes and the supernatants were used as enzyme source. The oxidation of catechol was measured from the reaction mixture containing two ml phosphate buffer (pH 6.5), 0.5 ml of enzyme extract and one ml of 0.01 M catechol. The absorbance was recorded at 495 nm at the interval of 30 seconds on UV-visible spectrophotometer (Shimadzu-1601). Proteins in 0.5 ml of enzyme extract were estimated and the enzyme activity was expressed as \( \Delta \text{OD min}^{-1} \text{ mg}^{-1} \text{ protein} \). The activity of enzyme was assayed as per the method mentioned above.

**Superoxide dismutase** [EC 1.15.1.1]

The enzyme assay was carried out by using Dhindsa et al. (1981) method. The enzyme extract was prepared by separately homogenizing one g fresh leaf tissues of native and invasive weeds in 10 ml of chilled 0.1 M phosphate buffer (pH 7.5), containing 0.5 mM EDTA. The extract after passing through cheese cloth was centrifuged in RC for 15 min. at 20,000 g. The supernatants served as enzyme source. The enzyme assay was carried out at 27 ± 2\(^\circ\)C and absorbance was recorded at 560 nm. The three ml reaction mixture contained 13 mM methionine, 25 mM nitroblue tetrazolium chloride (NBT), 0.1 mM EDTA, 50 mM phosphate buffer pH 7.8, 50 mM sodium bicarbonate and 0.1 ml enzyme. The reaction was started by adding two \( \mu \)l riboflavin and placing the tubes below 15 W fluorescent lamps for 15 min. Switching off of the light and covering the tubes with black cloth terminated the reaction. Tubes without enzyme developed maximum colour. A non-irradiated complete reaction mixture that did not develop colour was used as a blank. The absorbance was recorded at 560 nm and one unit of enzyme activity was taken as that quantity of
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enzyme, which reduced the absorbance reading to 50% in comparison with the tubes lacking enzyme.

Extraction, isolation and identification of allelochemicals of selected weeds

The roots, stems and leaves of both the weeds *Cassia* and *Synedrella* were used initially for the preparation of leachates. But it was noted that maximum leaching was since the better leaching of materials was observed from leaves, only leaves were used for further chemical analysis and studies on allelopathic activities.

Shade dried powdered leaf material of *Cassia* and *Synedrella* (5g) was refluxed with ethanol for 12 h. The leachate was filtered and residual solvent was completely dried under reduced pressure. The remaining procedure was followed as per the flowchart (Fig. 2.1). To detect phenols and neutral compounds, precoated aluminium TLC plates were used, which were developed with 10% ethanol in toluene as solvent system and were observed under UV lamp at 365 nm. The five different pink chromophoric glowing spots were marked and then developed by iodine crystals to determine the various compounds.

Detection of allelochemicals by HPTLC

The detection of different allelochemicals was done from air shade dried leaf samples of *Cassia* and *Synedrella*, which were extracted in different solvents by using the method of Harborne (1984).

Sample preparation for HPTLC analysis

For detection of allelochemicals the above samples of leaves of both the selected invasive weeds were extracted in different solvents like Methanol, Chloroform, Toluene, n-Hexane, and ethyl acetate. It was found that methanol had maximum percent extractive values, therefore for further studies methanol extracts were used.

1g of leaf samples were separately extracted in 10ml methanol and sonicated for 10 minutes and kept overnight. These extracts were filtered through Whatman No.1 filter paper. The filtrates obtained were used for TLC analysis (Harborne 1984). The allelochemicals were detected by using the derivatising reagents like methanolic sulphuric acid and Liebermanns Burchard reagent at 366 nm at 550 nm respectively. The data obtained were analysed using the HPTLC system (CAMAG) consisting of a
LINOMAT IV applicator and TLC Scanner-III, precoated plates of silica gel (E-Merck) G60 F254 (Padashetty 2007).

The details of HPTLC analysis are given below:

Instrument :- CAMAG HPTLC  
Application mode :- Linomat IV Spotter  
TLC Plate :- TLC Plate, Silica gel 60 F254  
Application position :- 10 mm  
From base :- 20 mm  
Plate width :- 200 mm  
Starting position :- 10 mm  
Band width :- 8 mm  
Space between :- 10 mm  
Sample volume :- 10 µl  
Mobile phase :- Toluene: Ethyl acetate (8:2)  
Development mode :- Manual  

**Method I** :- Universal reagent.  
Derivatising agent :- 10% methanolic sulphuric acid.  
Spectrum wavelength :- At 366 nm in fluorescence mode.  

**Method II** :- Detection of terpenoides, pungent and bitters essential oils.  
Derivatising agent :- Anisaldehyde- sulphuric acid reagent, TLC plate was sprayed with reagent, heated at 100°C and then evaluated in visible mode.  
Spectrum wavelength :- At 550 nm in Absorbance mode.  

**Method III** :- Detection of terpenoids (triterpnoinds), steroids,flavonoids  
Derivatising agent :- Liebermann -Burchard reagent, sprayed plate was heated at 100°C for 5-10 min and then inspected under UV light  
Spectrum wavelength :- At 366 nm in fluorescence mode.  

The data obtained was analyzed using Scanner II, CAMAG CATS 3 Software.

**Detection of allelochemicals by GC – MS method**

The shade dried and ground powder of whole plant (25 g) at full maturity stage was used for steam distillation with distilled water. The distillate (500 ml) was collected and repeatedly extracted with solvent ether. The solvent was removed and residue was found to be 0.10% and 0.08% by the weight of dried material for *Cassia uniflora* and *Synedrella nodiflora* respectively. The powder of both plants (25 g) was
refluxed with n-Hexane for six hours. The solvent was removed under reduced pressure. The steam volatile residue and hexane extract were analyzed by Gas Chromatography coupled with Mass Spectrometry.

Gas chromatography analysis (Agilent 6890N with FID) was performed using HP-5 capillary column. GC-MS analysis was performed using a Shimadzu QP 5050A mass spectrometer coupled to a Shimadzu 17A gas chromatograph fitted with a split-split less injector and a DB-5 fused-silica capillary column (30m x 0.25mm i. d., 0.25µm film thickness). Helium was used as the carrier gas at a flow rate of one ml/min. The injection port was maintained at 250ºC, and the split ratio was 1:40. Oven temperature programming was done from 50 to 280ºC at 10ºC/min, and it was kept at 280ºC for 5 min. Interface temperature was kept at 250ºC. Ionization mode was electron impact ionization and the scanning range was from 40 amu to 400 amu. Mass spectra were obtained at 0.5 sec. interval.

The identification of the chemical constituents was based on comparison of their mass spectra with those of authentic standards of a mass spectra library (Nist and Wiley 275.L Database) and the literature retention indices of the peaks on HP-5MS column with literature values, and explanation and certification of spectra (Lee et al. 1999).

Procedure for IR, NMR and MASS spectroscopy analyses

All solvents were distilled before use. Organic layers were dried over anhydrous sodium sulphate. Column chromatographic separations were carried out using column grade (60-120 mesh) silica gel. Thin layer chromatography plates used were prepared by spreading organic solvent suspension of silica gel (Silica gel G, procured from Merck Specialities Private Limited, Mumbai, India) uniformly over glass plates. The spots on TLC were visualized by exposing them to UV radiation or iodine vapours and / or spraying reagents followed by charring in an oven. Precoated alumina TLC plates (Merck) were used. The spots on TLC were observed under UV at 254 and 365nm wavelength. IR spectra were recorded on FTIR-8400S SHIMADZU (Fourier Transform Infrared spectrophotometer). NMR spectra were recorded on Bruker FT AC-200, Bruker MSL-300, Bruker DRX -500 and Varian Mercury Plus instrument operating at 24ºC using residual signal of non-deuterated solvents as internal reference. The following abbreviations were used – singlet = s,
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doublet = d, triplet = t, quartet = q, multiplet = m, doublet of doublet = dd etc. Mass spectra, were recorded on a Finnigan-Mat 1020 C mass spectrometer using ionization energy of 70 eV and LCMS were taken on LC-MS Perkin Elmer Applied Biosystems SCIEX- API3000 (ESI mode).

**Extraction, isolation and characterization of allelochemicals in *Cassia uniflora* by NMR**

Air shade dried and powdered material was extracted (10g) using acetone as a solvent at room temperature with continuous stirring for 12hrs. The material was then filtered. Acetone was removed under reduced pressure and extract was obtained as a sticky mass (0.438g, 4.38%). Extract was showing mixture of seven compounds of which two were major and both the compounds were UVactive with green and pink glow, when the TLC was run in 20% ethyl acetate in toluene as solvent system.

The crude extract was adsorbed (438mg) on silica gel (5g). Broad fractionation was carried out using nonpolar to polar solvents using magnetic stirrer for 4hrs. The fractionation of acetone extract was carried out using n-hexane (250 ml, A), acetone (400 ml, B), Ethanol (200ml, C), and methanol (200ml, D) (Table 3.19).

The hexane extract A showed a mixture of five components of which two were major. It was found that one component was UVactive and showed green fluorescence. The TLC was carried out in 100% Toluene. The mixture was insoluble in acetone and ethanol.

**Purification of ‘A’**

The compound was purified by repeated crystallization using mixed solvent system of hexane and ethanol to yield pure hygroscopic, amorphous, white powder (8mg, ‘A1’).

**Extraction, isolation and characterization of allelochemicals in *Synedrella nodiflora* by NMR**

Air shade dried powdered plant material (25g) was refluxed with ethanol (100ml) for 18h. Solvent was removed under reduced pressure to get
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crude extract (16.040%). The TLC was showing 6 major spots in 20% ethyl acetate – toluene solvent system of which one compound was UV active at 365nm. Broad fractionation (2.910g) was carried out using different gradient polarity solvents (Table 3.22). Total four fractions were collected, details of which are given in Table 3.22.

**Separation and purification of compound ‘A’ from *Synedrella***

Crude mixture containing compound ‘A’ was concentrated and kept at room temperature for 18h. White crystals were separated out. The compound was purified by ethanol. The pure compound (40 mg) showed single spot on TLC. It decomposed at 270°C. The probable structure of the compound was established by modern spectral data.

**Analyses of mineral constituents in leaf samples and rhizosphere soil of selected invasive and native weeds**

**Analysis of leaf samples**

**Estimation of nitrogen content by MicroKjeldahl method**

Ten field-grown plants of mung bean from control and each treatment were harvested along with roots on 45th day very carefully and the roots were thoroughly washed under tap water to remove the soil particles. The nodules were carefully collected and the fresh weight of nodule was recorded. The nitrogen content in leaf and nodule samples was determined as per MicroKjeldahl method.

1g of each sample was digested using 20 ml conc. sulphuric acid, to this five grams of sodium thiosulphate was added and heated for five minutes. The contents were cooled and a mixture of K₂SO₄ and catalyst (10:1 ratio) was added to the digestion tube. The digestion was carried out for one hour. After completion of digestion, the mixture was cooled and diluted to 100 ml with distilled water.

The distillation was carried out by adding ten ml of digest in vacuum jacket. In a conical flask, ten ml of 4% boric acid solution was taken containing bromocresol green and methyl red indicator, in which the condenser outlet of the flask was dipped. To the aliquot ten ml of 40%, NaOH solution was added. Five ml aliquot was distilled in the flask containing ten ml of boric acid.
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After completion of distillation, boric acid was titrated against N/200 H₂SO₄. Blank was run and titration was carried out to the same end point as that of the sample and the total nitrogen content was calculated as follows:

Mineral analysis by AAS method

The fresh leaf samples of Cassia and Synedrella were collected at mature stage from all the four sites, brought to the laboratory, cleaned and kept under shade, for drying at room temperature for four to five days. 1g completely dried samples were digested in triple acid mixture (nitric acid + perchloric acid + sulphuric acid in the proportion of 10:3:1). Final volume of each sample was made up to 100 ml with distilled water. The mineral constituents in native and invasive weeds were analyzed by AAS using Bhargava and Raghupati (1993) method.

Analyses of rhizosphere soil samples

The composite rhizosphere soil samples (100 g) of Cassia and Synedrella were collected at random from different sites at 20-25 cm depth and air-dried at room temperature. After thorough mixing, the dried samples were crushed gently in mortar with pestle and sieved through two mm sieve of nylon. Determination of pH and EC was done by using pH meter and conductivity meter (Elico). The mineral constituents like N, P, K, Ca, Mg, Zn, Cu and Fe were analyzed by using methods described by Rao (1993) and Gupta (1993).

Estimation of Phosphorus from soil sample

To 1g of soil sample, a small quantity of phosphorus free activated charcoal was added. It was kept on reciprocating shaker for 30 mins, after adding 50 ml Olsen’s reagent. This was filtered through Whatman No. 40 filter paper. Five ml of the filtrate was acidified with 2.5 M H₂SO₄ to get pH 5.0, to this 20 ml distilled water was added along with 4 ml of reagent B. The intensity of blue colour (after 10 min) was read at 730 nm on spectrometer (Rao 1993).

Estimation of Potassium

To 1g of soil sample, five ml 1 N Ammonium acetate (pH 7.0) was added. It was kept on reciprocating shaker for five min and then filtered through Whatman No.
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1 filter paper. The potassium content was determined with the help of flame photometer using the method of Rao (1993).

Estimation of Ca, Mg and micronutrients:

The soil sample was prepared as described for potassium and the contents of Ca, Mg, Zn, Cu, Mn and Fe were determined using Atomic Absorption Spectrometer (Perkin-Elmer 3100, USA).

Preparation of leaf leachates

The leaves of Cassia and Synedrella were collected randomly from all the sites, during the flowering stage and brought to laboratory, cleaned with distilled water and spread on filter paper for shade drying at room temperature. The composite samples of dry leaves were ground in Wiley Mill to pass through 2 mm sieve. 100g powdered leaves were soaked in 1000 ml distilled water for 24 h at 25°C and the leachate was filtered through Buchner funnel using Whatman filter paper no. 1. The filtrate was stored in refrigerator in amber coloured bottles to avoid degradation (Plate XI).

Preparation of leaf extracts

The powdered leaves of Cassia and Synedrella were soaked in distilled water for 24 h and then crushed in mixer-grinder. The extract was filtered as above and stored in amber coloured bottles (Plate XI).

Preparation of leaf residue

The powdered leaves of both the weeds, Cassia and Synedrella, were kept (after weighing) in nylon net bags (21×26cm size) with mesh size of two mm (Plate XI).

Measurement of p\text{H} and electrical conductivity of leaf leachates and extracts

The pH of different concentrations of leaf leachates of Cassia, Synedrella and their rhizosphere soils along with control soil was measured with the help of an instrument Elico LI - 610. For this the leaf leachates were prepared as mentioned above, while for the soil solutions, 10g of soil was dissolved in 25ml of distilled water and kept for half an hour then pH of the soil solution was determined. Electrical
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conductivity of supernatant soil solution and leachates was measured with Elico CM - 180 instrument at 20mS.

Biochemical analysis of residues before and after the treatments

The analysis of leaf powder samples of Cassia and Synedrella was carried out for measuring the weight of residues before and after the treatments to the test crop. The p$^{\text{H}}$ and EC of their solutions were measured with the help of above mentioned methods. The C : N ratio was calculated with the help of methods of mineral analysis methods mentioned above while the total phenolics in the residues were estimated as per the method of Farkas and Kiraly (1962).

Selection of test crops

Mungbean (Vigna radiata) var. Vaibhav and mustard (Brassica juncea) var. Seeta were selected as test crops.

Procurement of seeds of test crops

The certified seeds of mungbean var. Vaibhav and mustard var. Seeta were procured from the Director of Seed Farm, Mahatma Phule Agricultural University, Rahuri, Dist. Ahmednagar (M.S.).

Seed germination bioassay

Healthy seeds of mungbean and mustard were used for seed germination bioassay studies in seed germination chamber, using sterilized petriplates (9 cm dia) lined with germination paper. The seeds were surface sterilized with 0.02% aqueous Hgcl$_2$ for two minutes. Then the seeds were thoroughly washed with distilled water. Seed germination papers were thoroughly moistened (5ml.) with respective concentrations of leaf leachates of Cassia and Synedrella (2.5% - 20%), which were prepared by dilutions with distilled water. The seeds kept in distilled water were considered as control. Ten seeds were uniformly kept in each petriplate. The 2.0 ml leachates of respective concentration were added in each petriplate on third day. The petriplates were irrigated with the leachates only once. The treatments were replicated thrice.

Observations on percent seed germination (Prado et al. 2000), root and shoot length, root: shoot ratio, fresh and dry biomass and vigor index (VI) were recorded on
seventh day as per the method of Gupta et al. (1996). The seeds showing normal emergence of radical and plumule were considered as germinated. All the observations were recorded on ten randomly selected seedlings from each replication and treatment, by taking average values.

**Physiological and biochemical analyses of treated seedlings of test crops**

The physiological and biochemical changes in leaf leachates treated seedlings of mustard and mungbean along with control were analyzed at seventh day, according to the methods described previously.

**Experimental design of field trials**

The field experiments were conducted at the farm of Spicer Memorial College, Pune, Pune 411007 (M.S.) India. The test crops like *Vigna radiata* in *rabbi* and *Brassica juncea* in *kharif* seasons were grown under uniform conditions. All the experiments for mungbean were carried out in triplicate following FRBD design. The size of raised bed was two x two m and each plot had 20 – 25 plants per treatment. The distance between two plants was 15 cm and the distance between two adjacent plots was one meter. The required crop protection and agronomic practices were followed. The seeds of mungbean were sown in ridges. The distance between two plants was 15 cm and the distance between two rows was 30 cm. The meteorological data of this site was recorded (Tables 2.1, 2.2, 2.3).

**Applications of leachates and extracts to test crops**

The aqueous leaf leachates and extracts of *Cassia* and *Synedrella* were diluted with distilled water to get desired concentrations i.e. 1.5%, 2.5%, 5%, 7.5% and 10%.

Foliar applications of the leachates and extracts, 100 ml per plant for the field grown plants of mungbean and mustard were applied by hand sprayer method. Distilled water sprayed plants were considered as control. The leachates and extracts were applied on seventh DAS and continued up to 50% flowering/anthesis at the interval of seven days for mungbean and mustard respectively.

**Application of leaf residue to test crops**

The residue bags were buried in the soil in the vicinity of the root system of the test crop mungbean for about three months.
Analyses of physiological and biochemical parameters of leachates, extracts and residues treated test crops

Freshly harvested and randomly selected leaf samples (third leaf from top) were used for the physiological and biochemical analyses. Ten field-grown plants from each treatment and control were used for physiological and biochemical analyses by using the methods described previously.

Assay of nitrate reductase activity in leaf and root nodules

The *in vivo* anaerobic nitrate reductase (NR) assay was carried out by Sawhney et al. (1978) method. For this 1g freshly harvested leaf material and root nodules of mungbean plants were collected and cut into small pieces and transferred to test tubes containing five ml of assay mixture (2.5 ml of 0.1 M phosphate buffer-pH 7.2, 0.2 ml n-propanol and 2.3 ml DW). The tubes were vacuum infiltrated for 45 minutes in the dark at 30 °C. The reaction was terminated by keeping the tubes in boiling water bath for ten minutes. The assay mixture was cooled and one ml was used for developing colour with sulphanilamide and napthol ethylenediamine reagent for ten minutes. The optical density was measured on UV-visible spectrometer (Shimadzu-1601) at 540 nm.

Assay of antioxidant enzymes

The randomly sampled 1g fresh tissues of third leaf from the top of treated and control plants at 50% flowering/anthesis stage were homogenized in five ml 0.1 M phosphate buffer (pH-7). The extract was centrifuged in refrigerated centrifuge at 15,000 rpm for 20 minutes and the supernatant was used as enzyme source. The same enzyme source was used for assay of peroxidase, polyphenol oxidase and superoxide dismutase activity by employing the methods described previously.

Analysis of mineral constituents in rhizosphere soil

The composite rhizosphere soil samples (100 g) from ten randomly selected field grown mungbean of each treatment and control, before and after harvesting of crops were collected at 20 to 25 cm depth, air dried at room temperature. After thorough mixing, the dried samples were crushed gently in porcelain mortar with pestle and sieved through two mm sieve of nylon. Determination of pH and EC was
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done by using pH meter and conductivity meter (Elico). The mineral constituents of soil solution were analyzed by methods described earlier.

**Analysis of mineral constituents in leaf samples of mungbean**

The fresh leaf samples (third leaf from the top) of control and treated plants of mung bean and mustard at 50% flowering/anthesis stage were collected from ten randomly sampled plants. These leaf samples were cleaned and air-dried in the laboratory under shade at room temperature for four to five days. 1g completely dried leaf samples were digested in triple acid mixture. Final volume of each sample was made up to 100 ml with distilled water. The leaf mineral constituents of both the test crops were determined as stated above.

**Analyses of growth parameters**

The growth parameters in field grown treated and control plants such as height, number of leaves and number of branches per plant and leaf area etc were recorded at 50% flowering/anthesis stage by taking ten randomly selected plants from each treatment and control. The average values were recorded in tables.

**Analysis of root nodules**

The total number and fresh weight of nodules in treated and control of mungbean were determined from ten randomly selected plants in each treatment. The average values were recorded in tables.

**Analysis of yield contributing parameters**

The yield contributing parameters in mustard and mungbean such as number of pods per plant, length of pod, and number of seeds per pod, seed weight per ten pods and weight of 100 seeds were recorded for field grown plants from randomly selected ten plants in each treatment and control. The average values of the above parameters were recorded in the tables.

**Studies on cytotoxicity of selected invasive weeds**

**Preparation of leaf leachates for cytological studies**

For preparation of leaf leachates 100g shade dried material of both the plants was soaked in 500 ml of distilled water for 24 hours at $25\pm2 \degree C$ and the leachates
were filtered through Buchner funnel using Whatman filter paper No.1. These leachates were used as stock solution (20%), it was diluted to make different concentrations (5% to 20%). These leachates were stored in refrigerator in amber coloured bottles to avoid their degradation.

**Treatments**

About 25 equal sized rooted onion bulbs were treated with 5%, 10%, 15% and 20% leaf leachates of *Cassia uniflora* and *Synedrella nodiflora*, along with control (DW) at room temperature (25 ± 2 ºC). After 24 h the root tips were cut and fixed in FAA (5:45:50 v/v) for 24 h and preserved in 70% ethanol. Squash preparations were made from the treated and control roots as per the method of Sharma and Sharma (1980), which were examined under compound microscope and the cells were scored for mitotic abnormalities. The mitotic index (MI), relative division rate (RDR) and relative abnormality rate (RAR) were calculated by using following formulae --

1) $MI = \frac{\text{Total no. of dividing cells}}{\text{Total no. of cells examined}} \times 100$

2) $RDR = \frac{\% \text{ of dividing cells in treated root tips} - \% \text{ of dividing cells in control root tips}}{100 - \% \text{ of dividing cells in control root tips}} \times 100$

3) $RAR = \frac{\% \text{ of abnormal cells in treated root tips} - \% \text{ of dividing cells in control root tips}}{100 - \% \text{ of dividing cells in control root tips}} \times 100$

**Estimation of proteins from leachates treated onion root tips**

Randomly selected treated and control root tips were used (0.1g) for the analysis of soluble proteins by using the method of Lowry et al (1951).

**Analysis of larvicidal activity of selected invasive weeds**

**Preparation of leachates**

The leaves of *Cassia* and *Synedrella* were collected at flowering stage and brought to the laboratory, cleaned with distilled water and spread on filter paper for shade drying. The dried leaves were powdered and 100g powder was soaked in 1000ml distilled water for 24 hours at 25±2ºC and the leachates were filtered through Buchner funnel. It was stored in refrigerator in amber coloured bottles (to avoid degradation). These solutions were considered as stock (20%) from which leachates of various concentrations were made with distilled water.
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Procuring of mosquito larvae

The eggs of *Aedes aegypti* were obtained from Department of Entomology, National Institute of Virology, Pune. The eggs were kept in a tray with clean water for hatching. A pinch of dog’s biscuit powder was added for proper growth of larvae. The II instar larvae were used for experiment.

Treatments

The larvae were exposed to various concentrations of leachates (2.5 to 20%) in different bowls. The results were recorded for four days after every 24 hours interval, and LC$_{50}$ (Median Lethal Concentration of leachates) values were recorded.

Analysis of antimicrobial activities of selected invasive weeds

Procurement of pure strains of microorganisms

The pure strains of microorganisms were procured from department of biotechnology, University of Pune and also from National Chemical Laboratory, Pune.

Antibacterial bioassay

The test organisms used were *Bacillus subtilis* ATCC 6633 and *Escherichia coli*. Disc (methanolic extract) or well diffusion (aqueous extract) method was used for bioassay plant leachates. In this method, each 100 µl of 16 hrs old culture of *Bacillus subtilis* ATCC 6633 and *Escherichia coli* were spread separately on the antibiotic assay media no.3 and sterile paper disc (6mm diameter) or well (6mm in diameter) were, impregnated or filled, with test solution respectively. The plates were incubated at 28 °C for 16 hrs. The zone of inhibition around disc or well indicate the extract as bioactive materials (Miller et al. 2002).

Preparation of bioassay plates

The test organisms used was *Magnaporthe grisea*. For preparation of bioassay plates, a 25 ml vol. of sterile and molted and cooled corn meal agar was poured in to 100 cm sterile petri-dishes and allowed to solidify. After solidification, plates were preserved at room temperature and used after 2 days (Miller et al. 2002)
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Well diffusion assay

Well diffusion method was used for bioassay of plant leachates. In this method, an agar plug (6mm) of seven days old growing mycelial mat of *Magnaporthe grisea* was placed into the centre of corn meal agar plate and incubated at 28 °C for three days. Peripherally, about one cm from the margin of grown mycelial mat, wells (6mm) were bored and filled with test solution. The plates were incubated at 28 °C for three days. 1µg/ml of clarithromycin was added to the basal media to prevent the bacterial contamination (Franz and Harald 2000).

Disc diffusion method

Disc diffusion method was used to bioassay the methanolic extract of plant leacheates. In this method, an agar plug (6mm) of seven days old growing mycelial mat of *Magnaporthe grisea* was placed into the centre of corn meal agar plate and incubated at 28 °C for three days. Peripherally, about one cm from the margin of grown mycelial mat, disc impregnated with methanolic solution, dried in air was placed around the margin of mycelial mat and incubated at 28 °C for two days. The zone of inhibition around disc or well indicated the extract as bioactive materials.

Statistical analysis

The data were summarized as the pooled means of three replicates each over two years with standard deviation as the measures of variability. One-way ANOVA was used to compare the mean values as affected by different leachate concentrations. Duncan’s Multiple Range Test (DMRT) was applied at p<0.05 to compare the mean differences. The data were analysed by SYSTAT (ver.11) and Microsoft excel 2000.

For all the experiments on analysis of insecticidal activity of invasive weeds, LC$_{50}$ value and 95% confidence limits were calculated by Reid Munch method (Woolf 1968), using Microsoft Excel (7.0).

The data for analysis of cytotoxicity of invasive weeds were presented as means of three determinants (n=3) followed by standard error. One way ANOVA test was used to determine the significance of results. LSD at p = 0.05 was used to test significant difference of results between the means. The statistical analysis was carried out by using Sigmastat 3.5 (SYSTAT) and Microsoft Excel 2007.
The data from four sites with three replicates were summarized as pooled means after passing the covariance test with the standard deviation as the measure of variability. One way ANOVA was used to compare the mean values followed by DMRT at $p = 0.05$ to compare the mean differences. SigmaStat 3.5 and Microsoft Excel 2007 were used for the data analysis. Fisher’s LSD at $p = 0.05$ was used to test the significant difference between means (Ms Excel 2007).