MATERIAL AND METHODS
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Research Site:

a) Location and Topography:

Present investigation has been conducted in Department of Botany, D.V. Post-
Graduate College, Orai (Jalaun) which is situated at latitude 25° 59' N, longitude 79° 37'E
and is about 125 m above mean sea level in Bundelkhand region as well as Indian
Grassland and Fodder Research Institute, Jhansi located at 78° 35' E longitude, 25° 7' N
latitude and 275 m altitude from the mean sea level the northern part of Bundelkhand
region.

The region comprises seven districts of Uttar Pradesh viz., Jalaun, Hamirpur,
Mahoba, Banda, Chitrakut, Jhansi and Lalitpur and five districts of Madhya Pradesh viz.,
Datia, Tikamgarh, Chhatarpur, Panna and Sagar. The region is naturally bounded by the
river Yamuna in the north, range of Vindhyan Plateau in the south, river chambal in the
north-west and Panna-Ajaigarh ranges in the south-east.

The region has undulated topography that tends to become into a perfect level plain
towards north. About 1/3 northern part of the region is monotonously flat. Every where it
shows gentle undulating surface occasionally flat topped surface. The main rivers namely
the Betwa, Dhasan and Ken enter the alluvial plain in the north resulting in erosion on a
very large scale to form some of the most extensive and fantastic ravine land.

Bundelkhand plain is also known as Trans Yamuna plain and is
topographically devisible into three east-west running belts i.e. southern, central and
northern belts. The first belt narrower than the others and is confined along the bank of Yamuna in the form of high ground which represent the level of the ancient flood plain but which at present is badly cut up into deep ravines. Thus, the area in general exhibits subdued topography.

b) Geology and Soils:

The common rocks of the area are sand stones, lime stones and shales. The peculiar features of geomorphic interest are the long narrow serrated ridges termed as quartz, reefs and dolorites, dykes in this region. In the north-west and north-east, the geological system is covered by Ganga and Yamuna alluvial deposits in the form of an embayment.

Soil of Bundelkhand region may be conveniently grouped into the following categories (Regional Geography of India, 1960).

1. Upland soil (rocky soil)
2. Low land soil- black (mar, kabar), red and yellow (parwa, rankar) soil.
3. River i.ne soil (kachhar and tarai):

The most important soil groups of Bundelkhand are found in the northern low land. These are mar, kabar, parwa and rankar, formed partly in situ and partly by transporting agencies chiefly the streams.

Mar is calcareous soil predominantly blackish in colour mixed with lumps of Kankar and hence friable and aerated. Kabar on the other hand is highly diffused soil and is similar to mar in many physical characteristics. Parwa the best known variety of degraded red and yellow soil groups is well aerated, friable and receptive to irrigation and favourable
for various types of crops. Rankar is associated with flood plains subjected to gully and erosion so that calcium nodules are exposed at the sloping surfaces, rendering them unsuitable for cultivation. Thus on the basis a number of soil samples study, the soil is of medium textured and sandy loam to loam. The colour of the soil is light olive brown or olive brown which is slightly alkaline in reaction.

c) Climate and Vegetation:

The climate of the area is a dry sub-humid, tropical monsoonic with a year divisible into three seasons namely rainy (July-October), winter (November-February) and summer (March-June). The annual temperature is uniformly high over 25° C but the mean monthly values vary considerably (13.9° C mean minimum to 34° C mean maximum). The mean total annual precipitation is 1169 mm of which about 80% falls between July to October.

Bundelkhand region can be considered as an ecologically degraded area having about 0.7 million ha area occupying central position in the country. The original vegetation cover of the area has almost been removed for inhabitation and cultivation. Out of total area about 7.2% is under mixed dry deciduous type of degraded forests. Butea monosperma (Dhak), Salmolia malbarica (Seman), Boswellia serrata (Salai), Acacia nilotica (Babul) and A. catechu (Khair) are the dominant trees of natural vegetation. Apart from these Balanites aegyptica (Hingota), Carrisa carandus (Karondha) and Capparis aphylla (Karil) shares a good proportion of the floristic composition of the vegetation. Scrubs and grasses represent the secondary growth throughout the region. Besides it, various forest tree plantations were introduced during past three decades under afforestation programme by the Government of India.
Seeds (dispersal units) Collection and Germination:

Mature seeds (dispersal units) of the five range grasses were collected from the Central Research Farm of Indian Grassland and Fodder Research Institute (IGFRI), Jhansi as well as National Research Centre for Agro-forestry (NRCAF), Jhansi during September to December, 1994 and 1995. Dispersal units were cleaned, dried in sun and stored in Polythene bags at room temperature.

The diaspores (dispersal units) of Bothriochloa intermedia and Dichanthium annulatum grasses were a 'diad' while the diaspore of Chrysopogon fulvus was a 'triad' and the diaspore of Pennisetum pedicellatum was a 'bur' and the diaspore of Panicum maximum was a single unit.

1. The diaspore of 'diad' consisting of 2 spikelets, one sessile and fertile (hermaphrodite) possessing the grain and the other pedicellate and sterile staminate) e.g. B. intermedia and D. annulatum (Plate 6).

2. C. fulvus consisting of three spikelets one sessile and fertile (hermaphrodite) possessing grain, while the other two are pedicellate and sterile (staminate).

3. In P. pedicellatum the diaspore unit is a bur (in volucellate spikelet).

4. In P. maximum the diaspore is a single unit.

In the present programme two kinds of germination studies have been taken (i) with a single sessile spikelet in all the species except in P. pedicellatum where the bur was used and which will be referred as spikelets throughout these studies (ii) with seeds (caryopsis) obtained by removing the husk (glumes, lemma, palea etc.) mechanically, hence referred
PLATE 6: DISPERsal units (SPIKELETs) OF FIVE RANGE GRASSES

B. INTERMEDIA  P. MAXIMUM

C. FULVUS

P. PEDICELLATUM  D. ANNULATUM
to as seed. Thus in the case of B. intermedia, D. annulatum, C. fulvus and P. maximum, the germination units taken during the studies were the sessile spikelets or seeds (caryopsis). While in the case of P. pedicellatum the germination unit used was an intact spikelet (bur) or seed. Therefore, the term diaspora has been used for the whole dispersal units in this investigation.

The details of all the specific treatments are described for each experiment, but standard conditions were used for all germination tests.

The germination studies were conducted in petridishes on whatman germination test papers soaked double distilled water. The seeds/spikelets were kept in between two germination test papers replicated five times with 100 spikelets/seeds. These petridishes were then placed in germination cabinets of the seed germinator at 32± 1° C.

DORMANCY STUDIES:

Tests for seed germinability of freshly collected seeds (obtained from the freshly collected spikelets by dehusking) of all the five grasses were conducted within one month from the date of initial seed collection to ascertain, if the seeds required any dormant period or not.

SCARIFICATION STUDIES:

Seven treatments were imposed on fresh (upto one month old seeds) and nine months old spikelets. The treatments were: T1- control (no treatment); T2- Pre-chilling i.e. spikelets were moistened in petridishes and kept in refrigerator for 7 days at 5° C before germination test; T3- hot water: spikelets emerged in hot water at 70° C for 10 minutes before the germination test; T4-heat treatment: spikelets placed in oven at 60° C for 24
hours; T-5 ethanol: spikelets soaked in 95% ethanol for 10 minutes and subsequently washed before germination test; T6- potassium nitrate: spikelets moistened with 500 ppm solution of KNO$_3$ at the initiation of germination and subsequently with distilled water; T7- gibberellic acid. spikelets moistened with 500 ppm of GA$_3$ at the start of experiment and thereafter with distilled water. Germination counts were made at regular intervals and upto 14th days.

**EFFECT OF STORAGE ON SEED GERMINATION OF RANGE GRASSES:**

Test for seed germinability and viability were conducted at three monthly intervals upto 24 months for spikelets/seeds (seeds obtained by dehusking of spikelets at three months intervals).

**RATE OF GERMINATION:**

Nine months old spikelets/seeds (obtained by dehusking of 9 months old spikelets) were used to compare the rate of germination between spikelets and seeds.

**ISOLATION AND CHARACTERISATION OF GERMINATION INHIBITORS:**

For the purpose of isolation and characterisation of germination inhibitors, diaspores of grasses were used. The isolation and characterisation was conducted by extracting the compounds with suitable solvents followed by paper chromatography and colour reactions following Ibrahim and Towers (1960), Hais and Mecek (1963), Seikel (1964), Harborne (1967, 1973 a) and Markham (1982).

Preliminary investigations for the characteristics test for detection of phenolic compounds in the diaspores were conducted by testing the diaspores leachate (i.e. 10 gm of diaspores soaked in 200 ml of methanol for 24 hrs.) with phenolic reagents viz., alcoholic
ferric chloride (1 g of FeCl₃ dissolved in 100 ml of 95% ethanol), and diazotised sulphanilic acid (Table 1). According to Seikel (1964) 2-3 ml of methanolic extract was taken in a test tube and few drops of FeCl₃ were added. Production of blue or brown colour was noted as indication of presence of phenolic compounds.

Characteristic tests for the detection of phenolic compounds in methanolic extract by phenolic reagents revealed the presence of phenolic onium-ion (anthocyanins) in the diasores of B. intermedia and P. pedicellatum. In case of D. annulatum, C. fulvus and P. maximum no anthocyanin pigmentation was observed and diasores leachate gave a positive test for phenolics. Therefore, the following technique was adopted for further studies.

**Table 1: Sprays Used for Detecting Phenolic Compound on Paper Chromatogram (PC)**

<table>
<thead>
<tr>
<th>Sprays</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Diazotised sulphanillic acid</td>
<td>A 0.3% solution of sulphanillic acid in 8% HCl (25 ml) is mixed with 5% sodium nitrate solution (1.5 ml) just prior to use. The PC is sprayed with this and then with a 20% solution of sodium carbonate before drying. Most compounds with free phenolic hydroxyl group show as yellow, orange, or red to brown spots.</td>
</tr>
<tr>
<td>2. Diazotised p-nitroaniline</td>
<td>25 ml of solution of p-nitroaniline (0.3%) in 80%. HCl is mixed with 1.5 ml of 5% sodium nitrite solution just before spraying. The PC is sprayed with this and then with a 20% solution of sodium carbonate before drying. Most of the compounds shows as purple, pink or brown spots.</td>
</tr>
<tr>
<td>3. FeCl₃.K₄Fe(CN)₆</td>
<td>1% alcoholic solution of both the compounds is mixed in equal volume just prior to use. Most of the phenolic compounds show blue spots.</td>
</tr>
</tbody>
</table>

Ref: Seikel, 1964; Merkham; 1982.
ISOLATION TECHNIQUE:

ISOLATION OF ANTHOCYANIN FROM B. INTERMEDIA AND P. PEDICELLATUM DIASPORE:

Weighed quantity of diaspores (250 g) of B. intermedia (about 2-3 months of age) were first extracted (in soxhlet extractors) with non polar organic solvents such as petroleum ether and chloroform to remove non-phenolic substances viz., chlorophyll, waxes, fats, water soluble salts etc. (Seikel, 1964). Thereafter, the diaspores were extracted with methanolic HCl (methanol-HCl, 97:3 v/v). The methanolic extract was then filtered through whatman No. 1 filter paper and concentrated by distilling method on a water bath. The residue was now applied on a folded whatman No. 3 mm. Chromatographic paper, 8 cm from the side edge and 3 cm in from the last fold.

Table 2: Solvent Used for Paper Chromatographic Analysis of Phenolics

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Solvents Composition</th>
<th>Approximate running time</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAW</td>
<td>n-butanol:acetic acid: water (4:1:5) mixed thoroughly in a separating funnel), upper phase used</td>
<td>12 to 15 hrs</td>
</tr>
<tr>
<td>IBW</td>
<td>isopropanol:butanol:water (140:20:60)</td>
<td>-do-</td>
</tr>
<tr>
<td>Forestal</td>
<td>acetic acid:water:HCl (30:10:3)</td>
<td>6 to 8 hrs</td>
</tr>
<tr>
<td>Formic</td>
<td>formic acid : water : HCl (5:3:2)</td>
<td>-do-</td>
</tr>
<tr>
<td>15% HOAC</td>
<td>15% acetic acid</td>
<td>4 to 6 hrs</td>
</tr>
<tr>
<td>1% HCl</td>
<td>water:conc. HCl (97:3)</td>
<td>5 to 6 hrs</td>
</tr>
<tr>
<td>HOAC-HCl</td>
<td>acetic acid:HCl:water (15:3:82)</td>
<td>6 to 8 hrs.</td>
</tr>
</tbody>
</table>

Ref: Seikel, 1964; Harborne, 1967, 73 a; Markham, 1982.
(Fig. 1) and allowed to run in BAW (for solvent composition, see Table 2) in a chromatocab (Plate 7) by descending chromatography (Markham, 1982). The anthocyanin appeared as a clear discrete coloured band, which were then cut from the dried paper and the pigment was eluted with methanolic acetic acid (methanol containing 1.0% acetic acid). The elutes were collected and concentrated by repeating the process according to Harborne (1967 and 1973 a). The chromatographically purified pigments was used for the determination of Rf values in different solvents by descending chromatography on whatman No.1 chromatographic paper. Solvents used for anthocyanin pigment (glycoside) were BAW and 1% HCl (Table 1). A part of the chromatographically purified pigment was acid hydrolyzed with 2 NHCl for ½ hrs in a water bath and the anthocyanidin (aglycone) was collected in amylalcohol. Finally the identity of cyanidin (aglycone) was confirmed by co-chromatography with the authentic sample in Forestal, Formic and BAW.

In case of P. pedicellatum freshly collected diaspires (reddish purple in colour) were used for the extraction of anthocyanin pigment and the procedures given above for B. intermedia was also followed in this case for isolation of phenolic onium-ion.

**ISOLATION OF PHENOLIC COMPOUNDS FROM C. FULVUS, P. PEDICELLATUM, D. ANNULATUM, B. INTERMEDIA AND P. MAXIMUM:**

500 g of 10-15 months old diaspires were first extracted with petroleum ether in sponglet extractor to remove non polar and non phenolic substances viz., chlorophyll, waxes, fats, water soluble salts (Seikel, 1964). Thereafter, the used material was again extracted in hot MeOH: H₂O (9:1). The extract was concentrated to 50-60 ml by in vacuo distillation. The resultant aqueous extract was again extracted (in a separating funnel) with chloroform and the process repeated several times. The purified aqueous extract was now
PLATE 7: A CHROMATOGRAPHY CHAMBER
hydrolysed with 2N HCl on a water bath and filtered through whatman No. 1 filter paper. The filtrate was then extracted for ether and ethyl acetate soluble phenolic substances with the help of separating funnel. The ether and ethyl acetate soluble fractions were dissolved into 5% sodium carbonate solution, acidified to pH 2 and again extracted with ether and ethyl acetate, respectively (Ibrahim and Towers, 1960). Both the extracts were evaporated to near dryness and the residues were taken into a small amount of ethanol and combined.

The combined residue was now applied evenly to a whatman No. 3 chromatography paper, 3 mm diameter at a point about 8 cm from the side edge and 3 cm from the last fold which was folded as illustrated in Fig. 1 to permit the securing of the paper in a through for descending chromatography. The spotted paper was allowed to run in BAW for 15 hrs approximately in a chromatocab. The chromatogram was then trimmed off and refolded for descending chromatography in second dimension using 15% acetic acid for 5 to 6 hrs. The chromatogram was then viewed under ultraviolet light for spot detection with and without ammonia vapours. All the visible spots were pencilled in and eluted in 95% ethanol. Ethanol was evaporated and the spot residues were applied on whatman No. 1 chromatographic paper (No. 1 chromatography). The chromatograms were developed by descending chromatography in one dimension using BAW, and 15% acetic acid. Rf. (X100) of the spots were determined by colour reactions with diazotised sulphanillic acid, p-nitroaniline and FeCl₃. K₄ Fe(CN)₆ (Table 1). Finally the identity of the same compounds were confirmed by co-chromatography with the authentic samples (since identity of the remaining phenolics could not be confirmed as the standard samples of many of others were not available).
Fig 1 (A-B). Paper Chromatography Methodology.
BIOASSAY STUDIES:

The most widely used bioassay for allelopathic activity is the study of inhibition of seed germination and root as well as shoot growth. Therefore, bioassay studies were conducted to know the effect of water extract of the diaspor, on seed germination as well as root and shoot growth with the respective grass species and with two test species viz., radish (Raphanus sativus) and black gram (Vigna radiatus). Leachate obtained from all the respective grasses was diluted as 25, 50 and 100% concentration and one treatment was also kept as control (distilled water). Radish seeds have been widely used for bioassay studies owing to high and uniform germination and while black gram was also selected due to its rapidity of germination. Seeds of radish cv. Japanese white were obtained from commercial source while seeds of black gram were collected from farm of NRCAF, Jhansi.

The visible colours of leachate of C. fulvus, P: pedicellatum, D. annulatum, B. intermedia and P. maximum was light yellow, brown, yellow, dark brown and light brown, respectively (Plate 8).

The percentage inhibition of seed germination, root and shoot growth were computed as follows:

Percent(%) inhibition = 100x (N-n/N); where ‘N’ is the (%) germination or root growth or shoot growth in control and ‘n’ is the germination percent or root growth or shoot growth in the treatments.

STATISTICAL ANALYSIS

All the data recorded were put to statistical analysis by converting the percentage data in angular values (Panse and Sukhatme, 1967).
PLATE 8: AQUEOUS EXTRACTS OF DIA SPORE OF FIVE RANGE GRASSES