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Chemical Transformations and Biological Studies of Terpenoids Isolated from Essential Oil of *Cyperus scariosus*

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Cyperus scariosus is a potential medicinal herb belonging to the family *Cyperaceae*. The GC-MS analysis of the oil showed cyprene (18.57 %) as the major terpene present in it. Cyprene was isolated from the non-polar fraction of the oil using hexane as solvent and characterized using TLC and spectral techniques (IR and ^{1}H NMR). Cyprene was derivatized to cyprene epoxide by two methods *i.e.* using perbenzoic acid and epichlorohydrin. Further, the oil, it's polar fraction (dichloromethane), non-polar fraction (hexane), cyprene and cyprene epoxide were screened for their plant growth regulating property in case of wheat seedlings (HD 2967 and PBW 621). Complete germination was observed above 2.5 µg/mL of all the test fractions in both the cultivars. Moreover, cyprene epoxide was found to be the most effective in enhancing the length of roots and shoots. Seedling vigour index was calculated in order to analyze the enhancement shown by the oil and its various components on the seedlings.

Keywords: *Cyperus scariosus*, Cyprene, Cyprene epoxide, Plant growth regulation, Wheat.

INTRODUCTION

Cyperus scariosus is a medicinal herb and exhibits a wide spectrum of biological activities [1], which includes antibacterial and cytotoxic [2], antifungal [3], insecticidal [4] and antinociceptive [5], hepatoprotective [6], hypotensive and bradycardiac [7] properties. *C. scariosus* is widely distributed in India, especially in Chhattisgarh, Bihar, Orissa, West Bengal and Uttar Pradesh [8]. Extensive work has been carried out to show the plant growth regulating properties of terpenoids isolated from medicinal plants [9]. Among terpenoids lactones, conjugated sesquiterpenoid lactones, conjugated sesquiterpenoid ketones and their derivatives with α-methylene-γ-lactone moiety have shown enhancement in adventitious root formation in hypocotyl cuttings of *Vigna radiata* [10], bud break in grapevine cuttings [11] and sprouting in sugarcane cuttings [12].

A seed is the basic input in agriculture and a high quality seed is the basis of higher agricultural productivity. Quality in seed includes all physiological, biological, pathological and genetic attributes that contribute towards the final yield of a crop. Seed vigour is a concept describing several characteristics, which include rate and uniformity of germination and growth, tolerance to environmental stresses after sowing and retention of performance after storage. Seeds which perform well in some or all of these aspects are termed as high-vigour seeds [13]. It is generally recognized that the increase in seed dry weight improves seed vigour [14].

Wheat is the world's most widely cultivated food crop. The area sown under this crop is the largest among the area under various other cereal crops. It is eaten in various forms by more than one thousand million human beings and it makes a large contribution to the calories and proteins available to man than from any other food crop [15]. To date no work has been reported on the chemistry and plant growth regulating activity of *C. scariosus* in wheat seeds. The *C. scariosus* oil and the compounds present in it are believed to be responsible for plant growth regulator activity.

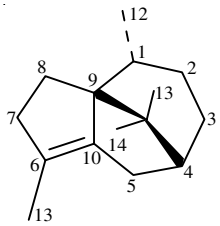
EXPERIMENTAL

IR spectra were taken in nujol on Perkin Elmer, Model RX-1 FT-IR spectrophotometer. ^{1}H NMR spectra were recorded with Bruker AC (400 MHz) as solutions (in $CDCl_3$) using tetramethylsilane (TMS) as internal reference. The IR and ^{1}H NMR spectra were taken at SAIF, Panjab University, Chandigarh. The chemical shifts are expressed in δ (ppm) values. GC-MS analysis was obtained from Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University, New Delhi, India. The wheat seeds were obtained

from Directorate of Seeds, Punjab Agricultural University, Ludhiana, India.

Column chromatography of *Cyperus scariosus* oil into non-polar and polar fractions: *Cyperus scariosus* oil (5 g) was subjected to column chromatography to fractionate it into non-polar and polar fractions. For column chromatography, the column was packed with 300 g of silica gel with 60-120 mesh size activated at 110 °C for 1 h. Oil was dissolved in hexane and then adsorbed on silica gel for 5-10 min. Column was eluted with hexane as solvent to obtain its non-polar fraction which gave one major spot on TLC plate along with other minor spots. Polar (dichloromethane) fraction gave a number of spots on TLC plate.

Isolation of cyprene from *C. scariosus* oil: Thin layer chromatography of non-polar fraction on silver-nitrate impregnated chromatoplates gave one major spot along with other minor spots. Major spot, after isolation with the help of column chromatography, was identified as cyprene (**1**) on the basis of spectral analysis when compared with literature [16].



Structure of cyprene (**1**)

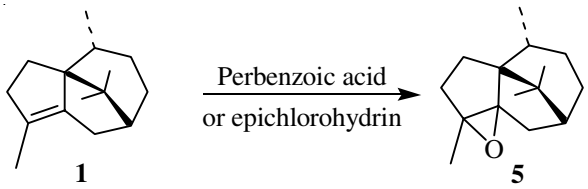
Epoxidation of cyprene (1)

Preparation of perbenzoic acid (4): Sodium metal (10.4 g) was added to methanol (200 mL) in small portions in a round bottomed flask fitted with a reflux condensor. The temperature of the flask was maintained at 0 °C using crushed ice. Solution of freshly recrystallized benzoyl peroxide (**2**) (100 g) was added to chloroform (200 mL, low temp.) in small portions with constant stirring at 0 °C. This solution was added to sodium methoxide in small portions with continuous stirring keeping the temperature 0 °C. The mixture turned milky after 5 min and was poured on crushed ice. The mixture was transferred to a separatory funnel and was allowed to remain undisturbed for 5 min. Aqueous layer was washed with chloroform (2 × 150 mL) to remove the last traces of benzoyl peroxide and methyl benzoate. The aqueous layer containing sodium benzoate (**3**) was then acidified with 450 mL of ice cold 1 N sulfuric acid (**Scheme-I**). Finally perbenzoic acid (**4**) was extracted from the acidified aqueous layer using chloroform (3 × 150 mL) and dried over anhydrous sodium sulfate. Perbenzoic acid (**4**) in chloroform solution was stored in refrigerator.

Determination of normality of perbenzoic acid solution: Sodium thiosulfate solution (0.5 N) was taken in a 250 mL conical flask and known amount of chloroform solution of perbenzoic acid (**4**) was added to it. An equal volume of dilute sulfuric acid and 1 g of KI was added to the flask. Then 1-2 g of Na_2CO_3 was added to the flask and covered with a glass lid for 1-2 min. The mixture was diluted with 10 mL water and few drops of sodium thiosulfate solution were added to it. Starch solution (2 mL) was added to the flask as an indicator to get the blue coloured solution. The solution was finally titrated with sodium thiosulfate solution with vigorous shaking till the blue colour of the solution was discharged [17].

1 mL of 0.1 M $Na_2S_2O_3$ ≡ 0.0069 g of perbenzoic acid

Epoxidation using perbenzoic acid: A solution of cyprene (**1**) (1.5 g) in chloroform (10 mL) was treated with an excess of perbenzoic acid solution in chloroform (**Scheme-II**). The reaction mixture was stirred continuously while the temperature was maintained at 0 °C with the help of an ice bath. The completion of reaction was checked by thin layer chromatography. The reaction mixture was diluted with chloroform, washed with saturated sodium thiosulfate and sodium bicarbonate solution, finally with brine and then dried over sodium sulfate. Evaporation of solvent afforded a thick yellow liquid (0.8 g), which was chromatographed over silica gel (100 g). Dichloromethane fraction afforded pure cyprene epoxide (**5**) and was identified by spectral analysis.

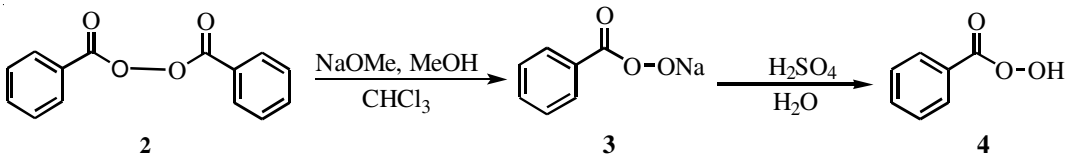


Scheme-II: Synthesis of cyprene epoxide (**5**) from cyprene (**1**)

Epoxidation using epichlorohydrin: Cyprene (**1**) (0.5 g) and epichlorohydrin, C_3H_5ClO (30 mL), were refluxed with continuous stirring for 13 h at 60-65 °C (**Scheme-II**). On completion of the reaction (TLC), the reaction mixture was filtered and extracted from chloroform. The organic phase was washed with 10 % NaOH (50 mL), water and then with brine. The organic phase was then concentrated and column chromatographed to obtain pure epoxy compound. Finally, the oil, its polar and non-polar fraction, cyprene (**1**) and cyprene epoxide (**5**) were used for the bio-efficacy studies as plant growth regulator in case of wheat seedlings.

Plant growth regulator studies

Seed germination and seedling establishment studies in *Triticum aestivum*: The seeds were germinated in petridishes (9 cm) lined with filter paper and moistened with solution of the oil, polar fraction, non-polar fraction, cyprene (**1**) and cyprene epoxide (**5**) at different concentrations along with distilled water as control. Ten seeds were planted per



Scheme-I: Synthesis of perbenzoic acid

petri-dish. The experiment was replicated thrice. The petri-dishes were incubated at 20 ± 2 °C. Petri-dishes were observed daily for 10 days for germination (radical protrusion of 2 mm). Primary root length and shoot length were measured at the end of ten days with a centimeter scale.

At the end of 10 days, seedlings were blotted dry and fresh weight was recorded. For dry weight determination, the seedlings were oven dried at 60 °C for 3 days in an incubator.

Preparation of stock solution: The stock solution (2000 µg/mL) of oil, its polar and non-polar fractions, cyprene (**1**) and cyprene epoxide (**5**) was prepared by dissolving each chemical (20 mg) in 1 mL of Tween 20 (polyoxyethylene sorbitan) and volume was made 10 mL with distilled water. The stock solution of 2000 µg/mL of each compound thus prepared on active ingredient basis and was kept in refrigerator till use. The required dilutions of 25, 20, 10, 5, 2.5, 1 and 0.5 µg/mL were subsequently made from the stock solution by adding distilled water as and when required.

RESULTS AND DISCUSSION

C. scariosus oil was obtained from laboratory stock and was reddish brown liquid. The physical properties of the oil were examined such as refractive index (1.39), specific gravity (1.508), viscosity (2.061 poise) and pH (3.3).

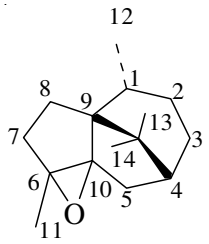
The essential oil was insoluble in water, sparingly soluble in hexane and completely soluble in acetone and ethanol. Thin layer chromatography of the *C. scariosus* oil showed seven coloured spots having R_f values of 0.95, 0.90, 0.83, 0.70, 0.63, 0.55 and 0.45. Gas chromatography-mass spectrometry (GC-MS) data of isolated *C. scariosus* oil showed the presence of cyprene (**1**) (18.57 %) as the major compound. Other minor compounds present were cyclopropazulen-7-ol, caryophyllene oxide, isolongifolen-5-one, longiverbenone, zierone *etc*. *C. scariosus* oil was subjected to column chromatography to fractionate it into non-polar (hexane) and polar fractions (dichloromethane). The TLC of non-polar fraction showed one major pinkish-brown spot having R_f = 0.95, along with other minor spots. However, the dichloromethane fraction showed six spots having R_f values 0.90, 0.83, 0.70, 0.63, 0.55 and 0.45.

Isolation of cyprene (1) from *Cyperus scariosus* oil: Column chromatography of non-polar (hexane) fraction of *C. scariosus* afforded major spot cyprene (**1**). The purity of the compound was checked by TLC and the structure was confirmed by spectral analysis.

The ^{1}H NMR ($CDCl_3$) spectrum of compound **1** showed singlets at δ = 1.62 ppm (C11-CH_3) which indicated the presence of tetrasubstituted double bond, 0.77 ppm (C13-CH_3) and 0.95 ppm (C14-CH_3). A doublet at 0.80 ppm (J = 6.4 Hz) was observed for the -CH_3 group at C12 position. The compound failed to show any signal corresponding to the olefinic protons, thus, it confirmed that the double bond was tetrasubstituted.

The IR analysis of compound **1** showed intense bands at 1382, 1372 cm^{-1} corresponding to C13 and C14 *gem* dimethyl group. A band was observed at 3078 and 1644 cm^{-1} corresponding to C-H stretching and C=C stretching of tetrasubstituted double bond, respectively.

Epoxidation of cyprene: The oxidation of terpenes is an important industrial application as epoxides are used as starting materials in the synthesis of commercially important materials for enhancing fragrance and flavor. Cyprene epoxide (**5**) was obtained from cyprene (**1**) by two different methods (**Scheme-II**). In first method, cyprene (**1**) was treated with chloroform solution of perbenzoic acid (**4**) taken in excess with continuous stirring. In another method, cyprene (**1**) was refluxed with epichlorohydrin for 13 h. In both cases, cyprene epoxide (**5**) was obtained as a thick yellow liquid. The purity of the compound was checked by thin layer chromatography (benzene:ethyl acetate, 19:1) having R_f = 0.65 and the structure was confirmed by spectroscopic analysis.



Structure of cyprene epoxide (**5**)

The ^{1}H NMR ($CDCl_3$) spectrum of compound **5** showed singlets at δ = 1.19 ppm (C11-CH_3), 0.74 ppm (C13-CH_3) and 0.90 ppm (C14-CH_3). It also showed a doublet at 1.00 ppm (J = 6.84 Hz) corresponding to C12-CH_3 group. The chemical shifts observed in case of cyprene epoxide (**5**) were downfield as compared to those in cyprene (**1**) because of deshielding occurred due to formation of epoxide ring. Further, the formation of epoxide was determined with the help of IR spectrum. The IR analysis of cyprene expoxide (**5**) showed the presence of bands at 1277 cm^{-1} corresponding to C-O stretching and 889 and 803 corresponding to C-O bending of epoxy group. The intense bands corresponding to *gem* dimethyl group were found at 1378 and 1316 cm^{-1}.

Evaluation of *Cyperus scariosus* oil and its components for plant growth regulator activity: After the successful derivatization of cyprene (**1**) to cyprene epoxide (**5**), *C. scariosus* oil, its non-polar and polar fractions, cyprene (**1**) and cyprene epoxide (**5**) were screened for their plant growth regulation studies. For initial screening of the oil, its fractions, isolated compound and its derivative, we preferred to use those seeds which can give results within 7-10 days *e.g.* wheat, mung beans *etc*. Therefore, two different varieties of wheat (*Triticum aestivum*), HD 2967 and PBW 621 were selected. The effect of oil, its non-polar and polar fractions, cyprene (**1**) and cyprene epoxide (**5**) were studied on root length, shoot length, fresh weight, dry weight and seedling vigour index.

Observations: The different parameters such as fresh weight, dry weight, root length and shoot length were observed in control and are listed in Table-1.

The following observations were recorded in case of wheat seedlings treated with the oil, its fractions, isolated compound and its derivative:

Germination percentage: Germination percentage of the seeds germinated was calculated using the formula:

$$\text{Germination (\%)} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds kept}} \times 100$$

TABLE-1
PHYSICAL PARAMETERS FOR
HD 2967 AND PBW 621 IN CONTROL

Parameters	HD 2967	PBW 621
Fresh weight	0.235 g	0.208 g
Dry weight	0.063 g	0.054 g
Root Length	11.00 cm	10.00 cm
Shoot Length	12.00 cm	11.50 cm

The germination percentage increased with the increase in concentration for each fraction. At higher concentration (above 2.5 µg/mL) complete germination was observed in all the cases.

Number of roots: The number of roots were found to be four in each case at each concentration of the compounds as well as in control in both cultivars.

Length (shoot and root): Lengths were measured using a centimeter scale. Shoot length was measured from the base till tip of the uppermost leaf. Root length was measured from the base till tip of the longest root. Both shoot and root length increased with the increase in concentration of each compound. Cyprene epoxide (**5**) was found to be the most active in enhancing shoot (Table-2) and root length (Table-3) at higher concentrations in both varieties. The oil and its polar fraction also showed a good potential in enhancing the shoot and root length.

Fresh weight of seedling: The seedlings were blotted dry on the 10th day of the experiment and fresh weight was recorded. Likewise, fresh weight of the seedlings showed a linear relationship with the concentration of the compounds. The oil, its polar fraction and cyprene epoxide (**5**) increased the fresh weight of the seedlings more efficiently as compared to cyprene (**1**) and the non-polar fraction. Cyprene epoxide (**5**) showed a maximum effect in increasing the weight of the seedlings (Table-4).

Dry weight of seedling: The seedlings were oven dried at 60 °C for 3 days and then the dry weight was recorded. A similar trend was observed in determination of dry weight of seedling as in case of fresh weight. Cyprene epoxide (**5**) was found to be the most effective in increasing the dry weight of the seedling (Table-5).

Seedling vigour index (VI): Seedling vigour index (VI = % germination × total seedling dry weight) also increased with the increase in concentration in all the cases. The results of seedling vigour index showed that the oil, its polar and

TABLE-2
EFFECT OF OIL AND ITS VARIOUS COMPONENTS ON SHOOT LENGTH (cm)

Conc. (µg/mL)	Oil		Non-polar		Polar		Cyprene		Cyprene epoxide	
	HD 2967	PBW 621	HD 2967	PBW 621	HD 2967	PBW 621	HD 2967	PBW 621	HD 2967	PBW 621
0.5	10.50	9.40	9.75	9.23	10.95	10.20	10.10	9.76	13.07	12.06
1.0	11.26	10.30	10.23	9.87	11.37	11.30	11.64	11.45	13.33	12.09
2.0	12.04	11.27	11.43	10.34	12.56	12.42	12.30	12.23	13.58	12.16
2.5	12.90	12.14	11.96	11.54	13.28	13.14	12.98	12.51	13.96	13.00
5.0	13.81	13.23	12.77	12.35	14.03	14.00	14.50	13.07	14.67	14.23
10.0	14.73	13.61	13.46	12.83	15.69	14.70	15.08	13.57	15.96	14.83
20.0	16.53	14.00	14.87	13.06	17.28	15.70	15.50	15.20	17.53	15.96
25.0	17.48	15.61	15.82	14.23	17.74	16.33	16.23	15.47	18.47	17.20

TABLE-3
EFFECT OF OIL AND ITS VARIOUS COMPONENTS ON ROOT LENGTH (cm)

Conc. (µg/mL)	Oil		Non-polar		Polar		Cyprene		Cyprene epoxide	
	HD 2967	PBW 621	HD 2967	PBW 621	HD 2967	PBW 621	HD 2967	PBW 621	HD 2967	PBW 621
0.5	12.25	11.97	10.10	9.50	13.68	12.34	11.04	10.38	14.20	11.92
1.0	13.00	12.40	10.76	10.33	15.42	13.42	12.95	11.36	16.50	12.48
2.0	15.80	14.50	11.96	11.56	16.66	14.03	14.28	12.61	17.02	14.30
2.5	16.30	15.00	12.63	12.15	17.46	14.33	14.97	13.33	17.80	15.50
5.0	17.50	15.70	13.02	12.83	18.86	15.76	15.46	14.37	19.60	16.20
10.0	18.20	16.00	13.76	13.06	19.53	16.33	16.55	15.90	20.91	16.40
20.0	20.40	16.30	14.06	13.58	20.06	17.46	18.60	16.73	22.30	18.02
25.0	21.72	16.70	14.77	13.90	21.16	18.23	19.98	17.48	22.50	19.60

TABLE-4
EFFECT OF OIL AND ITS VARIOUS COMPONENTS ON FRESH WEIGHT (g)

Conc. (µg/mL)	Oil		Non-polar		Polar		Cyprene		Cyprene epoxide	
	HD 2967	PBW 621	HD 2967	PBW 621	HD 2967	PBW 621	HD 2967	PBW 621	HD 2967	PBW 621
0.5	0.252	0.241	0.202	0.192	0.249	0.239	0.238	0.236	0.279	0.246
1.0	0.284	0.253	0.216	0.208	0.273	0.247	0.257	0.243	0.297	0.259
2.0	0.293	0.262	0.228	0.214	0.286	0.251	0.264	0.249	0.304	0.265
2.5	0.307	0.269	0.249	0.232	0.304	0.256	0.272	0.252	0.316	0.278
5.0	0.321	0.276	0.253	0.241	0.318	0.268	0.292	0.261	0.353	0.284
10.0	0.348	0.284	0.272	0.248	0.339	0.274	0.315	0.269	0.381	0.292
20.0	0.369	0.298	0.292	0.253	0.358	0.286	0.333	0.277	0.392	0.326
25.0	0.398	0.311	0.342	0.267	0.376	0.291	0.364	0.286	0.431	0.348

TABLE-5
EFFECT OF OIL AND ITS VARIOUS COMPONENTS ON DRY WEIGHT (g)

Conc. (µg/mL)	Oil		Non-polar		Polar		Cyprene		Cyprene epoxide	
	HD 2967	PBW 621	HD 2967	PBW 621	HD 2967	PBW 621	HD 2967	PBW 621	HD 2967	PBW 621
0.5	0.066	0.054	0.069	0.056	0.070	0.061	0.065	0.053	0.072	0.058
1.0	0.068	0.058	0.073	0.059	0.073	0.064	0.068	0.057	0.079	0.062
2.0	0.074	0.062	0.078	0.064	0.079	0.067	0.069	0.062	0.082	0.067
2.5	0.079	0.065	0.083	0.069	0.082	0.070	0.075	0.064	0.087	0.072
5.0	0.086	0.069	0.086	0.073	0.089	0.076	0.081	0.068	0.094	0.076
10.0	0.093	0.073	0.092	0.079	0.097	0.081	0.087	0.073	0.106	0.083
20.0	0.099	0.079	0.093	0.084	0.103	0.089	0.096	0.078	0.112	0.087
25.0	0.108	0.084	0.098	0.089	0.119	0.093	0.104	0.082	0.121	0.095

TABLE-6
SEEDLING VIGOUR INDEX (VI)

Conc. (µg/mL)	Oil		Non-polar		Polar		Cyprene		Cyprene epoxide	
	HD 2967	PBW 621	HD 2967	PBW 621	HD 2967	PBW 621	HD 2967	PBW 621	HD 2967	PBW 621
0.5	6.60	4.64	6.90	4.81	7.00	5.24	6.50	4.53	7.20	4.98
1.0	6.80	4.98	7.30	5.07	7.30	5.54	6.80	5.97	7.90	5.33
2.0	7.40	5.95	7.80	5.88	7.90	6.17	6.90	6.02	8.20	5.76
2.5	7.90	5.98	8.30	6.62	8.20	7.20	7.50	6.14	8.70	6.62
5.0	8.60	6.90	8.60	7.30	8.90	7.65	8.10	6.80	9.40	6.99
10.0	9.30	7.30	9.20	7.90	9.70	8.10	8.70	7.30	10.60	8.30
20.0	9.90	7.90	9.30	8.40	10.30	8.90	9.60	7.80	11.20	8.70
25.0	10.80	8.40	9.80	8.90	11.90	9.30	10.40	8.20	12.10	9.50

non-polar fractions, cyprene (**1**) and cyprene epoxide (**5**) have improved the performance of the seeds in terms of germination (Table-6).

Statistical analysis: The statistical analysis for the plant growth regulator activity of the oil, its polar and non-polar fractions, isolated compound cyprene (**1**) and cyprene epoxide (**5**) was carried out. The results were analyzed using Factorial CRD. The interactions between concentrations (A), compounds (B) and varieties (C) were analyzed to study the enhancement of root length, shoot length, fresh weight, dry weight and seedling vigour index in case of both the varieties (HD 2967 and PBW 621). The results are as follows:

Wheat cultivar HD 2967: The value of coefficient of variation was found to be 8.2432 for shoot length, 7.7591 for root length and 1.494 for dry weight. The interaction between A and C was found to be significant (least significant difference (LSD) = 0.943) amongst all other factors in case of shoot length. All the interactions *i.e*. between concentrations, compounds and variety with respect to control (*i.e.* without treatment) were significant in root length and dry weight. No significant results were obtained for the interaction between compounds and/or concentrations with respect to the variety for seedling vigour index. Thus, it can be concluded that the effect of concentration was significant with respect to variety as compared to control (*i.e.* without treatment) and all the compounds behave similarly on increasing the shoot length.

Wheat cultivar PBW 621: The coefficient of variation was 8.6021 and the only significant interaction was between A and C (LSD = 0.9375) for shoot length whereas all the interactions *i.e.* between concentrations, compounds and variety with respect to control (*i.e.* without treatment) were significant for root length. The seedling vigour index for the variety was significantly different (LSD = 0.3294) as compared to control (*i.e.* without treatment) and a significant interaction was observed between the concentrations and the variety (LSD = 0.9316).

Conclusion

The epoxidation of cyprene (**1**) with epichlorohydrin was more convenient as compared to that with perbenzoic acid (**4**) as well as the yield of the reaction was more in case of epichlorohydrin. In general, the oil, its polar and non-polar fractions, cyprene and derivatives enhanced the growth of the wheat seedlings. The decreasing order of activity at different concentrations was as follows:

Cyprene epoxide (**5**) > Polar fraction > *C. scariosus* oil > Cyprene (**1**) > Non-polar fraction

Thus, it can be concluded that there exist a linear relationship between the oil, its polar and non-polar fractions, cyprene (**1**) and cyprene epoxide (**5**) with the plant growth regulating activity as with the increase in concentration, the length of the roots and shoots increased. Moreover, it is very interesting to note that cyprene epoxide (**5**) has been found to be the most active in promoting the root and shoot length and are promising plant growth regulators.

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