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Radiation Chemistry

RADIATION TREATMENT AND DIOSGENIN CONTENT IN *DIOSCOREA BULBIFERA* L.

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The irradiated *Dioscorea* tubers were grown in the field and the resultant mutants were studied for three consecutive vegetative generations. Morphological, cytological, anatomical and phytochemical studies were performed from both control and treated series. Morphological and cytological changes observed were insignificant, but conspicuous alterations in number and size of stomata, number of epidermal cells and stomatal index were observed. A comparative chromatographical study shows qualitative changes in steroidal sapogenins, terpenes, and alkaloidal content within the treated series as compared with the control. A new steroidal sapogenin compound appeared in the first vegetative generation after irradiation and remained consistent up to third generation. Moreover, intensity reaction shows a remarkable increase in diosgenin content in the irradiated material, which is maximum in the second and third vegetative generations.

INTRODUCTION

Dioscorea is an important genus of medicinal value, containing a large amount of steroidal sapogenin compounds, especially diosgenin. Diosgenin is a precursor of cortisone. A number of steroidal hormones including progesterone can be prepared from this compound (Marker 1940). It is used in various human disorders as well as in the control of fertility. Different centres are engaged in exploring new sources of diosgenin from various plant species. The bulk of the compound is obtained from different species of *Dioscorea* viz., *deltoidea, prazeri, composita, floribunda* and *mexicana.* Many other genera were found to contain steroidal sapogenin. Recently, *Costus speciosus* has been discovered as a new source of diosgenin.

Under the tropical conditions of West Bengal, two species of *Dioscorea* are rather common viz., *alata* and *bulbifera.* Both of them produce bulbils. Their growth is much more luxuriant than any of the other species of *Dioscorea.* Of these two, *D. bulbifera* has a very low diosgenin content (Barua *et al.* 1956), and in *D. alata* it is almost absent (Barua *et al.* 1954). Since, none of the species, which yield important sources of diosgenin, can be grown successfully under conditions prevailing here, it was thought worthwhile to investigate the extent to which the diosgenin content can be improved in *D. bulbifera* through radiation treatment. Such mutants, if obtained, would provide an important source of diosgenin as well as a useful material to breed with other

commercially important species of *Dioscorea,* because profuse growth and presence of bulbils are found during the propagation of *D. bulbifera* without requiring any special treatment.

Materials and Methods

Tubers of *D. bulbifera*, of 7 to 9 cm diameter, were irradiated with 15 k rad X-ray using a Radon X-ray machine, 100kvp, 11.2 mA, at a distance of 15 cm with an output of approx. 770 r min. The control and irradiated tubers were planted in earthenware pots. A duplicate set was also grown in the field for further propagation.

For the extraction of sapogenins, alkaloids and terpenes in the first year, a portion was removed directly from each irradiated tuber while the remaining portion was allowed to grow. Subsequently, samples were taken from the same tubers after growing for each year. Each yearly growth of the tubers was considered as a vegetative generation. The changes in morphological characters of the plants, chromosomes, stomatal index as well as size and number of stomata were observed after irradiation for three consecutive vegetative generations (i.e. M_1, M_2 and M_3) and compared with those of control plants.

Cytological study of control tuber was carried out from the young root tips. They were pretreated with saturated solutions of *p*-dichlorobenzene and aqueous aesculine (1 : 1), at 10-12°C for 2½ hr and fixed in propionic ethanol (1 : 3) for 1 hr, followed by 15 min hydrolysis in N HCl at 58°C; stained overnight with 2% propionic orcein and squashed in 45% propionic orcein. The root tips of irradiated tubers were fixed and stained following the same schedule without pretreatment.

Stomatal studies were made from the lowermost leaves of the 6 weeks old control and irradiated plants.

The sapogenin compounds were extracted by the method of Chakravarti *et al.* (1970). Chromatograms were prepared following Stahl's classical method of thin layer chromatography. The plates were kept in a separation chamber after application of spots, containing chloroform : methanol (99 : 1) as solvent, till the solvent covers 15 cm path over the plate. The spots were developed by spraying the chromatograms with Leibermann-Burchard reagent and heated at 60°C for half an hour. These chromatograms were then exposed under uv light for identifying fluorescent compounds; fluorescing or quenching zones were marked by pricking the outer margin with a needle and then transferred to the tracing paper. The Rf values of the different compounds were calculated.

Extraction of diosbulbine was carried out according to the method followed by Joshi and Rane (1969). The total chloroform extracts before separation of diosbulbine crystals were studied chromatographically. The crystals of diosbulbine were isolated and weighed. The chromatograms were sprayed with Ehrlich's reagent, and documentation of chromatograms was preserved.

Alkaloids of the control and irradiated plants were extracted from the dried, powdered tubers with petroleum ether in a soxhlet extractor. Different alkaloids were separated from the total extract chromatographically using a procedure similar to the one followed for sapogenins. The spots were developed with Dragendorff's reagent (Bregoff *et al.* 1953), iodine vapour, fluorescence was studied and Rf values were calculated.

OBSERVATIONS

Morphological data

The germination of irradiated tubers was delayed in comparison to the untreated one, but the shoot length increased more quickly than that of the latter. The nodal distances, petiole length , and size of the leaves decreased in the first generation, and other deformities related to leaf shape were also noted during the subsequent vegetative generations of irradiated plants. Early flowering in the irradiated material was recorded.

Cytological data

In control plants the somatic chromosome number was 2n=40. Cytoplasm was very dense due to heavy inclusions and raphide crystals. But in irradiated materials, cytoplasm was comparatively clear and raphide crystals were almost absent. Fragments, stickiness, laggards were also absent and somatic chromosome number remained unaltered in the irradiated material. Pollen mother cells could not be studied since all plants were female.

Anatomical data

The stomata of the control and treated *D. bulbifera* were studied from the lowermost leaves of the six weeks old plant, counted from the date of the emergence of shoot from the tuber. In *D. bulbifera*, stomata were present only in the lower surface of the leaves. The epidermal cells were rectangular or polygonal and arranged variously. The anticlinal walls were straight or slightly sinuous. Stomata were diffuse without any specific arrangement. Multicellular trichomes were present on the abaxial side of the leaves. Stomata were not accompanied by any recognizable subsidiary cells; in majority of cases they were surrounded by three such undifferentiated cells, often resembling the cruciferous type. Pant and Kidwai (1966) described these as tricytic stomata. Rarely, paracytic and tetracytic types of stomata were also observed in this material.

During preparation of tables and texts the terms, M_1, M_2 and M_3 have been used to refer the first, second and third vegetative generations after irradiation.

The number of stomata increased considerably in the M_1 as compared to control, and at the same time, number of epidermal cells also increased, with a decrease in size. The increase of stomatal number was slight in the M_2. It decreased sharply in M_3 with a sudden fall in the number of epidermal cells in the same generation. The stomata were elliptical in the control, but as their length decreased sharply and breadth showed slight decrease, in M_1 and M_2 they became rounded. Again in M_3, the stomata reverted to the original elliptical form. A remarkable increase of the stomatal index was observed in the M_1 followed by a gradual decrease in the subsequent generation i.e. M_2 and M_3 (Table I).

Phytochemical data

Comparative chromatographical studies of sapogenins, diosbulbine and alkaloids of control and irradiated plants were performed mainly qualitatively by spraying the chromatograms with different specific spray reagents. Diosgenin and diosbulbine were

TABLE I

Average stomatal frequency, size and index in control and irradiated D. bulbifera

Material	No. of stomata in the microscopic field (15.2 cm^2)	No. of epidermal cells in the microscopic field (15.2 cm^2)	Stomatal index	Length of stomata (in μ)	Breadth of Stomata (in μ)
Control	16.5	108.2	13.31	33.0	20.8
M_1	39.5	157.6	20.04	28.0	16.8
M_2	40.0	192.0	17.24	30.8	18.2
M_3	20.0	107.5	15.68	32.2	19.6

identified through the mixed melting point and mixed thin-layer chromatography of authentic and isolated compounds. The unidentified compounds were differentiated from each other by their respective Rf values, colour reaction with specific spray reagent and property of fluorescence.

The visual intensities of the spots were tabulated with plus symbols in tables; ++++ represents maximum intensity, and + very faint staining.

Eight different steroidal sapogenins were observed in the control material of *D. bulbifera* (Table II), although five of them including diosgenin, were present only in an extremely low quantity. In M_1, out of these eight sapogenins, only two could be spotted together with a significant amount of a third "new" sapogenin spot. This compound persisted also in the following generations. There was considerable increase of the diosgenin content in the M_1, M_2 which persisted in M_3 as well.

TABLE II

Relative staining intensities, colour reaction and fluorescence of steroidal sapogenin obtained from control and irradiated D. bulbifera

Rf values	Colour developed with Leibermann-Burchard reagent	Uv Fluorescence	Intensity of staining			
			Control	M_1	M_2	M_3
0.23	Greenish-blue	Q^1	++	–	–	–
0.37	Chocolate-brown	Q	+	–	–	–
0.48	Yellow	+	+	–	–	–
0.53	Reddish-brown	+	++++	+	++	+
0.56	Violet (Diosgenin)	+	++	+++	++++	++++
0.65	Chocolate-brown	+	++	–	–	–
0.73	Canary yellow	+	+++	–	+	–
0.85	Chocolate-brown	+	+++	–	+	–
0.94	Violet	+	–	+++	+++	+++

Q^1 = quenching spots

A comparison of the diosbulbine content was made by estimating the amount of this compound present in 100 g dry tuber tissue (dried after a preliminary boiling treatment). This showed the presence of 1 mg in the control, complete absence in the M_1, about 1.9 mg in the M_2 and very low amounts in the M_3 generations.

The other compounds in the chloroform extract were possibly terpenes. Two of them disappeared completely (Rf 0.11 and 0.55) in M_2 and M_3 (vide Table III). The compound at Rf 0.11 was present in very low amount in M_1. A new compound appeared in M_1 at Rf 0.31, persisted in the M_2 and disappeared again in M_3 generation.

TABLE III

Relative staining intensities and colour reaction of terpenes obtained from control and irradiated D. bulbifera

Rf values	*Spots developed after spraying with Ehrlich's reagent	Intensity of stain			
		Control	M_1	M_2	M_3
0.06	Brown	+++	++	++	+
0 11	Chocolate brown	+++	+	—	—
0.19	Grey	++	++	++	—
0.31	Pale green	—	+++	+++	—
0.45	Pink (Diosbulbine)	++	—	++++	+
0.55	Brown	+++	—	—	—
0.93	Yellow	+++	+	++	+++

*All the compounds were iodine-positive.

Comparative chromatographic studies revealed that in the M_1, all the 5 alkaloids (seen to be present in the control) decreased quantitatively, whereas some of them increased in M_2 (e. g. the compounds at the 0.57 and 0.81 Rf values). The alkaloid at Rf 0.81 again decreased in M_3 generation but the other one at Rf 0.57 remained same as in the M_2. A new alkaloid appeared in the M_3 at Rf 0.87 (vide Table IV).

TABLE IV

Relative staining intensities, colour reaction and fluorescence of alkaloids obtained from control and irradiated D. bulbifera

Rf values	*Spots developed after spraying with Dragendorff's reagent	UV** Fluorescence	Intensity of stain			
			Control	M_1	M_2	M_3
0.1	Green	Q	+++	++	+	—
0.25	Green	Q	+++	++	+	—
0.36	Pale green	Q	+++	++	++	+
0.57	Pink	F	++++	++	+++	++
0.81	Orange	Q	+++	++	+++	+++
0.87	Greenish-yellow	F	—	—	—	+++

*All the compounds were iodine-positive.

**Q, quenching spots; F, fluorescing spots.

A deep pink coloured compound at Rf 0.67, which was iodine positive, did not fluorescence, was Dragendorff's negative and originally present in the species, had completely disappeared in the M_1 and M_2 generations, but reappeared in a low quantity in M_3 generation. A yellow compound at 0.93 Rf point was seen in the M_1 in low quantity which was absent in M_2 and again reappeared in M_3. The nature of these two colouring compounds is not yet identified.

Discussion

In the present investigation X-ray treatment of tubers has been resorted to in the place of seeds. The objective was to circumvent the problems of seed germination as well as to secure rapid results with the mutagen treatment. Such a method, if successful, could open up a new avenue for obtaining a broad spectrum of mutations within a short period. The investigations carried out were based on the growth of the tubers in three successive seasons. Seeds obtained so far will be grown in the coming year. Recently, Martin *et al.* (1974) have reported stimulation of *D. alata* tuber growth by gamma irradiation.

It is true that the changes induced either at the chemical or at the morphological level cannot be attributed precisely to the effect on a single gene, since the tissue represents a mass of cells on which different gene loci are affected by X-rays. However, consistency of effect, in successive vegetative generations, if any, was sought for so that it may be utilized effectively for their medicinal value.

An analysis of the results so far secured indicates that there has been a significant increase in the number of stomata and epidermal cells in the first two vegetative generations. On the other hand, in M_3 there has been a reversion more or less to the original state. Increase in the number of stomata is associated with decrease in size and directly correlated with the number of epidermal cells. The decline in M_3 generation might have been due to the elimination of the epidermal cells and consequently of the number of stomata in competition with larger epidermal cells and stomata of larger size.

The chromatogram obtained for the different steroid sapogenins shows a broad spectrum in the control. In M_1, M_2 and M_3 however, there has been a significant decrease in the spectra or complete absence of most of them. An additional sapogenin occupying a different Rf point altogether has been consistently found in three successive vegetative generations. Similar consistency has been noted with diosgenin. In control, the diosgenin content was found to be very low, and there had been a significant increase in diosgenin content in all the three successive vegetative generations. Since this has been of uniform occurrence in all the generations, the effect may be considered as quite consistent and permanent. Efforts are now being directed to make a quantitative analysis of the content to confirm the results obtained from colour intensity reactions. Moreover, the plants being female, may be utilised for crossing with other species of *Dioscorea* having high diosgenin content. Experiments are in progress in this direction.

In addition to sapogenins, the alkaloids as well as the terpenes, particularly diosbulbine, the diterpene, were also analyzed to find out their correlation, if any, with the sapogenins. The procedure followed for the analysis of alkaloids is rather simple and that of sapogenin quite complex. It was therefore thought worthwhile to analyze the former

for the sake of comparison. In the event of a positive correlation, the alkaloid content can be used as a marker for the sapogenin content as well. However, in the relationship between terpenes and alkaloids, as the table would show, no such correlation could be obtained.

The present investigation clearly indicates that irradiation has been able to induce stable changes in the somatic tissue of *D. bulbifera*; this technique may be successfully exploited for increasing the diosgenin content. Furthermore, the plant produced from the irradiated tubers may be used for crossing with other species of high diosgenin content.

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ERRATUM

The folio numbers of pages 160 and 161 have been interchanged. Please therefore read page 161 as 160 and *vice versa.*