Influence of gamma radiations on levels of kaempferol in cultures of *Pisum sativum*

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Abstract: Genetically pure seeds of *Pisum sativum* variety *pusa harbhajan* were inculcated under aseptic conditions on revised murashige and Skoog’s medium supplemented with 1 ppm of 2,4-D and 1% agar. Four weeks old cultures were exposed to gamma radiations for three hr in replicates of five. Dose of radiations selected for treatment were 2000, 2400, 2800 and 3200 R. Cultures of different dose were harvested after four weeks, dried and subjected to extraction for flavonoids by well established method. Kaempferol was identified through TLC, PTLC MP and IR spectral studies whereas quantitative estimation was carried out using spectrophotometric method. Maximum amount (0.10 mg g\(^{-1}\)) was observed in the sample treated by 2800 R, followed by sample treated by 2400 R, 3200 R (0.05 mg g\(^{-1}\)) and 2000 R (0.03 mg g\(^{-1}\)). However remarkable increase in kaempferol content was observed when compared with control (0.008 mg g\(^{-1}\)) in all doses of gamma radiations.

Key words: Flavonoid, Gamma radiation, *Pisum sativum*, Tissue culture

Introduction

Plant tissue cultures have now emerged as an alternative to plants which have acquired world-wide dimensions in improving genetic potential of plants and their preparative use in methodologies. Cultures have been proved to retain biosynthetic capacities, as various compounds and enzyme systems of plants are found to be present in cultures too (Nag, 1976). Review of literature reveals that lot of work has been done in the field of secondary metabolites from tissue culture (Das, 1980; Vasil, 1980; Bajaj, 1981; Khanna et al., 1988; Kumar and Khanna, 1994; Drew and Van-Staden, 1995). Secondary metabolites although have been reported from tissue culture but substantial amount of compounds, so far has not been reported except for few viz shikonin from *Lithospermum erythrorizon* and ginsang from *Panax ginsang*.

Efforts have also been made time to time by various workers to increase the content of medicinally important compounds in tissue culture where effect of various factors and precursors on production of secondary metabolites have been studied (Singh et al., 2000; Mahana and Singh, 1971; Jain and Agarwal, 1987; Chauhan and Kumar, 2007).

In the present study, effect of gamma radiations on the production of kaempferol in tissue culture of *Pisum sativum* var *pusa harbhajan* has been studied.

Materials and Methods

Genetically pure seeds of *Pisum sativum* variety *pusa harbhajan* were soaked in distilled water for 24 hr which were then transferred to sterilized petri plates lined with water soaked cotton. After six days epicotyl region of germinated seeds were transferred to revised Murashige and Skoog’s (RT) medium, supplemented with 2,4-D and 1% agar.

Based on earlier studies (Kumar et al., 1989) cultures of maximum growth index (4 weeks old) was selected for gamma radiation treatment. Experiment was set using four weeks old cultures in four sets of five flasks each. Flasks of four sets were irradiated to 2000, 2400, 2800 and 3200 R doses of gamma radiations respectively for 3 hr using Co\(^{60}\) gamma cell irradiator (available in the Department of Radiotherapy, SMS hospital Jaipur).

Tissue was allowed to grow for four weeks to keep the uniformity of time. After four weeks tissue of each set was harvested separately and dried at room temperature till a constant weight was achieved.

Tissue of each set was then subjected to extraction for flavonoids following the method of Subramanian and Nagarajan (1969), re-extracted successively with petroleum ether, ether and ethylacetate. Petroleum ether fraction was discarded due to its being rich in fatty substances. Ether and ethyl acetate fractions of each sample were extracted for free and bound flavonoids respectively. Ethyl acetate fraction of each of the samples was hydrolysed by refluxing with 7% H\(_2\)SO\(_4\) for 2 hr. The extract was filtered and again extracted with ethyl acetate in separating funnel. The ethyl acetate layer was washed with distilled water to neutrality, dried and weighed.

Chromatography (Harborne, 1984; Ramawat and Merillon, 2000) qualitative:

Thin layer chromatography: Glass plates coated with silica gel and activated in an oven at 100°C for 30 minutes were used for TLC. Ether and hydrolysed ethyl acetate fractions were applied separately along with the standard reference compound of kaempferol. Solvent system used was benzene, acetic acid and water in the ratio of 125:72:3. Prominent spot coinciding with
Fig. 1: Infrared spectra of standard and isolated kaempferal
reference kaempferol (Rf 0.86) was observed in ethyl acetate fraction, which was further confirmed by spraying the plates with 5% ethanolic ferric chloride solution.

Preparative thin layer chromatography (PTLC): Ethyl acetate fractions of different samples were used for PTLC and the spot coinciding with reference kaempferol (Rf 0.86) was collected from about 200 developed and unsprayed glass plates along with the adsorbent (silica gel). The compound was eluted with methanol, filtered dried and rechromatographed to test the purity of the compound in each case.

Each isolate from different sample was crystallized separately and subjected to melting point and infrared spectral studies using potassium bromide pellets (Fig. 1).

Quantitative: Quantitative estimation of kaempferol was carried out colorimetrically with the help of spectrophotometer following the method of Mabry et al. (1970). Concentration of kaempferol in various test samples was studied in the regression curve on the basis of optical densities measured and concentration in each gram of test sample was calculated.

Results and Discussion

Kaempferol (Rf 0.86; Uv fluorescent - bright yellowish blue, ammonia - deep yellow, FeCl₃-brown; m.p. 270-273°C) was identified in different tissue samples of *Pisum sativum* var *pusa harbhajan*. The characteristic IR spectral peaks were found to be super imposable with that of standard reference compound (Fig. 1).

Free form of kaempferol was not observed in ether fraction. However bound form of kaempferol was observed to be maximum (0.10 mg g⁻¹) in the extract of tissue exposed to 2800 R, followed by the tissue exposed to 3200 R, 2400 R (0.05 mg g⁻¹) and 2000 R (0.03 mg g⁻¹). Increase in kaempferol was observed in all cases when compared with control (0.008 mg g⁻¹) showing in Table 1.

Effects of various precursors and mutagens on production of secondary metabolites in tissue culture of different plants have been studied (Heble et al., 1971; Stohs et al., 1975; Khanna et al., 1988). The present study is an effort in the same direction where effect of gamma radiation on the production of kaempferol content in tissue of *Pisum sativum* has been studied for the first time.

Results obtained in the present study reveal that as the gamma radiation dose increases there is slight increase in the concentration of kaempferol up to 2800 R but thereafter increase in dose to 3200 R results decrease in kaempferol concentration.

Indiscriminate use of plants for medicinal purposes has posed problem of fast exhausting plant resources. International efforts are therefore being made to develop methods other than traditional ones for the storage maintenance, conservation and exchange of germplasm, also to find out an alternate source for the production of pharmaceutically important metabolites and if possible in higher amount and at lower cost. The focus of the present study is the application of tissue culture technology for the production of an important metabolite (kaempferol) from tissue culture of *Pisum sativum* var *pusa harbhajan* in higher amount by the use of gamma radiations. In the present scenario when pharmaceutical companies are mushrooming up, medicinal plants are at the verge of extinction due to uncontrolled harvesting, to be used as raw material for the production of important drugs. The present study where efforts have been made, not only to use tissue culture, instead of whole plant for extraction of important metabolite, but efforts have also been made to increase its content per gram, which is of great significance.

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References


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**Table 1:** Kaempferol content in control and gamma irradiated tissue of *Pisum sativum* var *pusa harbhajan*

<table>
<thead>
<tr>
<th>Dose of radiations (R)</th>
<th>Kaempferol content (mg g⁻¹)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.008</td>
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<tr>
<td>2000</td>
<td>0.03</td>
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<tr>
<td>2400</td>
<td>0.05</td>
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<tr>
<td>2800</td>
<td>0.10</td>
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<td>3200</td>
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