

## ***Agrobacterium rhizogenes*-mediated transformation of Witloof chicory – *In vitro* shoot regeneration and induction of flowering**

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Stable transformation and expression of transgene was achieved in Witloof chicory (*Cichorium intybus* L. cv. Lucknow local) using *Agrobacterium rhizogenes*-mediated system. Transformation frequencies varied with use of different types of strains of *A. rhizogenes*, concentration of *A. rhizogenes* and age of explants. The transfer of *rolA* gene into chicory genome was confirmed by PCR analyses. The supplementation of putrescine at 40 mM level with 2'-3'-*O*-isopropylidene adenosine (2-iP) (2 mg l<sup>-1</sup>) and, gibberellic acid (GA<sub>3</sub>) (0.5 mg l<sup>-1</sup>) to MS medium resulted in increased shoot multiplication and shoot growth in untransformed plants compared to the transformed plants of *C. intybus*. Putrescine at 40 mM level also resulted in flowering in both the systems on the 28th day, in untransformed and transformed plants. The supplementation of AgNO<sub>3</sub> at 40 µM level to the untransformed axillary buds of *C. intybus* on MS media with 2-iP (2 mg l<sup>-1</sup>) and GA<sub>3</sub> (0.5 mg l<sup>-1</sup>) resulted in higher shoot multiplication (36.9 ± 2.63 shoots per culture) and higher growth of shoots (7.82 ± 0.76 cm) in comparison to transformed ones for shoot multiplication (11.6 ± 0.89 shoots per culture) and growth of shoots (3.2 ± 0.24 cm). Moreover, 40 µM AgNO<sub>3</sub> treated cultures showed *in vitro* flowering on the 28th day in both the systems. The information generated in terms of transformation protocols, *in vitro* morphogenesis including flowering will make genetic transformation easier for chicory crop improvement.

TUMOUR-inducing plasmid (Ti) of *Agrobacterium tumefaciens* causes crown gall tumours, whereas root-inducing (Ri) plasmid of *A. rhizogenes* causes hairy roots on susceptible dicotyledonous plants<sup>1</sup>. Root induction is due to stable integration of the Ri-TDNA (transferred DNA) into the plant genome and its subsequent expression<sup>2</sup>. Ri transformed roots can be cultivated *in vitro* on hormone-free media and in some species regenerated into plants<sup>3</sup>. Plants can be regenerated from hairy root cultures either spontaneously from roots or by transferring roots to hormone-containing media. These transformed plants show an altered phenotype called hairy root syndrome, which despite some variable traits has characteristic features in

several plant species<sup>4</sup>. This altered phenotypic behaviour of the transformed plants includes, alteration in the basic developmental process like flowering, reduced apical dominance and plagiotrophic roots<sup>4</sup>, which are sexually transmitted as a set of dominant traits<sup>4</sup>.

*Cichorium intybus* L., the source for chicory of commerce belongs to Asteraceae family, and has been cultivated successfully in India. It is gaining global attention due to its industrial utility as a coffee additive, and for important phytochemicals<sup>5</sup>. We have shown earlier that exogenous putrescine and silver nitrate (AgNO<sub>3</sub>), influence morphogenesis in chicory shoot cultures<sup>6</sup>. Putrescine and AgNO<sub>3</sub> influenced morphogenesis with respect to shoot multiplication and *in vitro* flowering. Under these treatments, the shoot apices flowered without vernalization. This was also evident by the polyamine inhibitor studies carried out with the chicory shoot explants<sup>6</sup>. We have also shown that morphogenesis in shoots of chicory with respect to flowering and shoot multiplication was governed by the interplay between polyamine and ethylene biosynthesis<sup>6</sup>.

This paper reports production of transformed roots using different strains of *A. rhizogenes* and regeneration of transformed plants from such transformants. The stable transfer of transgenes has been shown by polymerase chain reaction (PCR) amplifications.

Seeds from ten different plants of same cultivar of *C. intybus* L. cv. Lucknow local were obtained from Banthara Research Station, National Botanical Research Institute (NBRI), Lucknow. The seeds were washed in running tap water and were surface sterilized by rinsing them in 70% ethanol for 10 s followed by surface sterilization in aqueous solution of 0.1% (w/v) HgCl<sub>2</sub> for 3 to 5 min in sterile deionized water. The MS basal medium<sup>7</sup> was supplemented with 3% sucrose (Hi media, India). The pH of the medium was adjusted to 5.8 ± 0.1 after gelling with 0.8% agar (Hi media, India). The gelled medium was autoclaved at 1.06 kg cm<sup>-2</sup> pressure and 121°C for 5 min. The seeds were inoculated onto MS basal medium and incubated at 25 ± 2°C under cool light (4.41 Jm<sup>-2</sup> s<sup>-1</sup> 18 h day<sup>-1</sup>). Axillary buds-derived 10 ± 2-day-old seedling cultures of untransformed *C. intybus* were excised, and were transferred to the MS medium with 2'-3'-*O*-isopropylidene adenosine (2-iP) (2 mg l<sup>-1</sup>), gibberellic acid (GA<sub>3</sub>) (0.5 mg l<sup>-1</sup>), putrescine (10–50 mM) and AgNO<sub>3</sub> (10–50 µM) for shoot multiplication.

Putrescine was obtained as its hydrochloride from Sigma Chemical Co, USA, and was incorporated into the medium in the concentration range of 10–50 mM after filter sterilization using 0.22-µm filter (Sartorius Ltd). AgNO<sub>3</sub> was obtained from Qualigens Co Ltd, India, and was added to the medium after filter sterilization using 0.22-µm filter (Sartorius Ltd). All other chemicals were of analytical grade and solvents of HPLC grade.

*A. rhizogenes* LMG-150, A20/83 and AZ/83 (mannopine-type strains) were obtained from P. I. J. Hooykaas,

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Clusius Laboratorium, Rijks Universiteit, Leiden, The Netherlands. Bacterial colonies were cultured for 3 days on solid YEM medium<sup>8</sup>. Transformed roots (hairy roots) were initiated by inoculating the wounded hypocotyls of 2–10-day-old seedlings of *C. intybus* with *A. rhizogenes* strains. Hairy roots were found to appear within 10 days of infection. Roots of  $3 \pm 0.2$  cm size were excised and immediately transferred to 40 ml MS basal medium containing carbenicillin ( $500 \text{ mg l}^{-1}$ ) in 150 ml conical flasks. Transformed roots were periodically subcultured at three days interval in antibiotic-containing medium. The roots were subsequently transferred to MS basal liquid medium and incubated in dark on a rotary shaker at 90 rpm and maintained at  $25 \pm 2^\circ\text{C}$ , to obtain axenic hairy root cultures.

Hairy root cultures derived from LMG-150, which showed beneficial effects over the hairy roots derived from A20/83 and AZ/83 in terms of faster growth rate and increased coumarin production (data not shown) were screened for spontaneous shoot regeneration and morphogenetic studies.

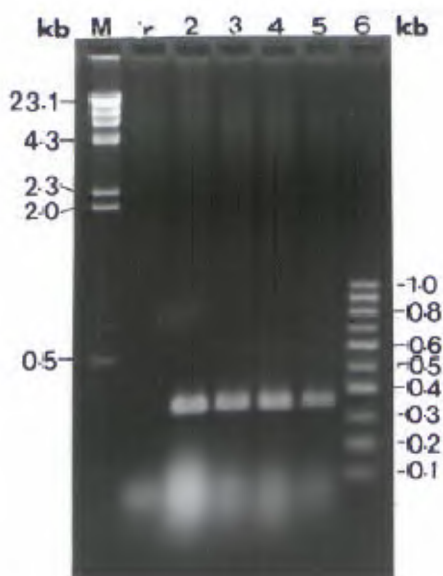
Hairy roots derived from LMG-150 strain were subjected to hormonal stress, wherein transformed root segments (3–4 cm in length) were subjected to various combinations of growth regulators for shoot regeneration. The resultant transformed shoots were excised and were cultured in the MS medium containing 2-iP ( $2 \text{ mg l}^{-1}$ ),  $\text{GA}_3$  ( $0.5 \text{ mg l}^{-1}$ ) with exogenous supplementation of

putrescine ( $10\text{--}50 \text{ mM}$ ) and  $\text{AgNO}_3$  ( $10\text{--}50 \text{ }\mu\text{M}$ ) to study the effect on *in vitro* shoot multiplication and flowering.

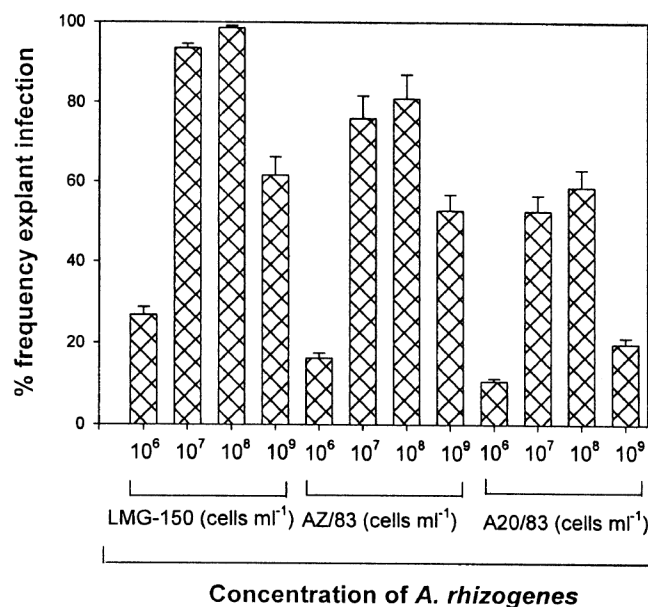
Total DNA was isolated according to Dellaporta *et al.*<sup>9</sup>, from the original hairy root clone (derived from the strain LMG-150), and from the two transformed plants (T1 and T2) and untransformed shoots of chicory. Plasmid DNA from *A. rhizogenes* strain LMG-150 was used as a positive control. Isolated DNA was analysed by PCR for *rolA* gene. The oligonucleotide primers used to amplify *rolA* gene on the T-DNA of *A. rhizogenes* (LMG-150) were designed according to Hamill *et al.*<sup>10</sup>, and obtained from Bangalore Gene-I India Pvt Ltd. The primers designed to amplify *rolA* were 5'-AGAATGGAATTAGCCGGACTA-3' and the reverse primer 5'-GTATTAATCCCGTAGGT-TTGTTT-3'.

PCR for *rolA* was carried out by amplification under the following conditions: Initial denaturation at  $94^\circ\text{C}$  for 4 min and then denaturation at  $94^\circ\text{C}$  for 1 min, annealing at  $55^\circ\text{C}$  for 1 min and extension at  $72^\circ\text{C}$  for 1 min for 35 cycles, with a final extension at  $72^\circ\text{C}$  for 5 min. The amplicons were analysed by electrophoresis on 2% agarose gel along with *EcoRI/HindIII* and 100 bp molecular markers<sup>12</sup> (Figure 1).

Of three strains of *A. rhizogenes*, viz. LMG-150, A20/83 and AZ/83 used in transformation studies, it was observed that LMG-150 at a concentration of  $10^8 \text{ cells ml}^{-1}$  and a co-cultivation of 3 days gave the maximum frequency explant infection compared to the other two strains used in the study (Figure 2). Hypocotyls of 2-day-old seedlings infected with  $10^8 \text{ cells ml}^{-1}$  of LMG-150 gave the maximum frequency of explant infection compared to the other explant of a different age (Figure 3). Of the three strains of *A. rhizogenes* tried, it



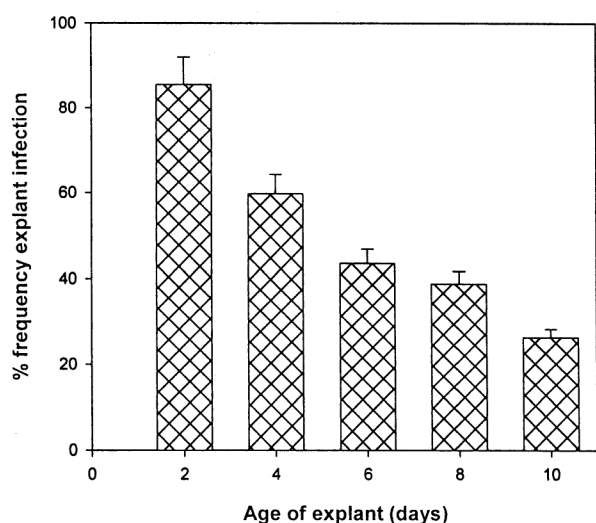
**Figure 1.** Agarose gel analysis of *rolA* (~360 bp) PCR amplicons from transformed hairy roots and shoots of *C. intybus* derived after infection with *Agrobacterium rhizogenes* (LMG-150). M  $\lambda$  *EcoRI/HindIII* marker. Lane 1, Untransformed shoots of chicory; Lane 2, Hairy root culture of chicory; Lane 3, Transformed shoots of chicory (T1); Lane 4, Transformed shoots of chicory (T2); Lane 5, Plasmid DNA from *Agrobacterium rhizogenes* (LMG-150); Lane 6, 100 bp DNA ladder.



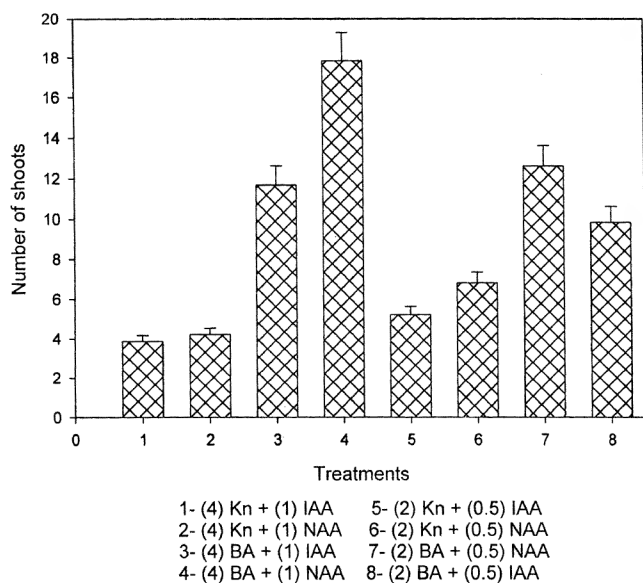
**Figure 2.** Effect of concentration of different types of *A. rhizogenes* strains on percentage frequency explant infection in *C. intybus*.

was seen that the hairy roots derived from LMG-150 were fast-growing and hence was selected for the further studies. Of the various growth regulator combinations tried, it was observed that the MS medium containing BAP (6-benzylaminopurine) ( $4 \text{ mg l}^{-1}$ ) with NAA ( $\alpha$ -naphthalene-acetic acid) ( $1.0 \text{ mg l}^{-1}$ ) resulted in maximum number of shoots ( $18.2 \pm 1.3$ ) after  $14 \pm 2$  days of culture period (Figures 4 and 5 a, b).

Of the treatments, 40 mM putrescine level to axillary shoot buds of untransformed plants (Tables 1 and 2) resulted in maximum shoot multiplication ( $34.8 \pm 2.61$  shoots per culture; Figure 5 c) as well as growth of shoots ( $7.69 \pm 0.57 \text{ cm}$ ), compared to the same treatment to the

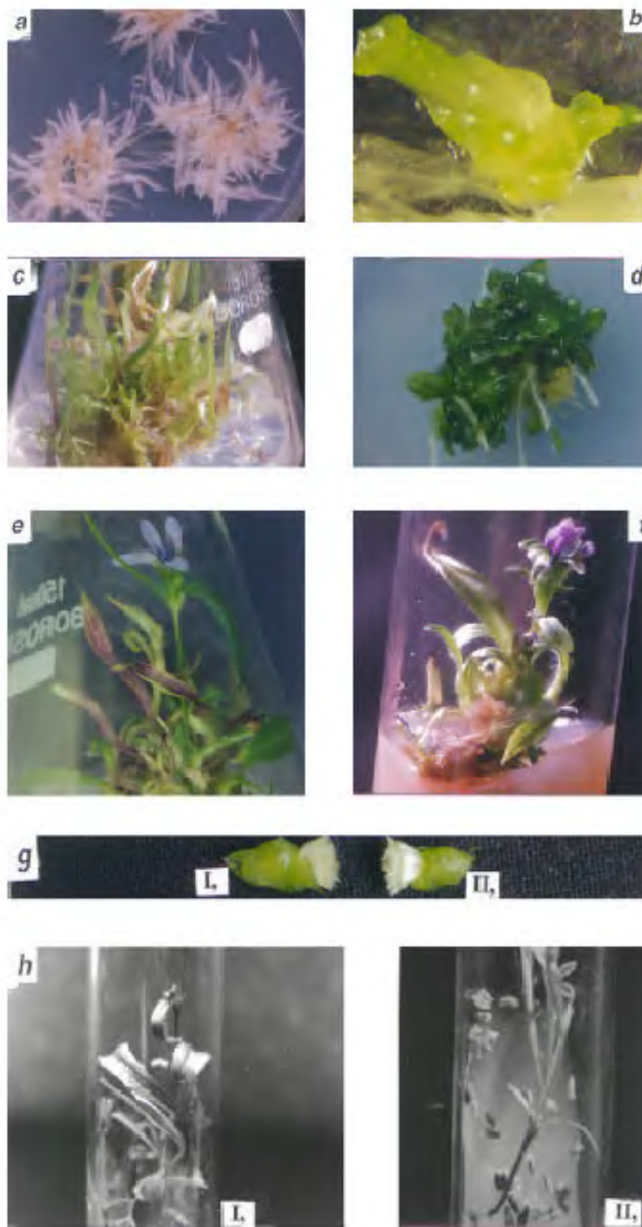


**Figure 3.** Effect of age of explant on percentage frequency explant infection with *A. rhizogenes* (LMG-150).



**Figure 4.** Effect of various concentrations of growth regulators in MS medium for spontaneous regeneration of shoots from hairy root cultures of *C. intybus*.

axillary shoot buds of transformed plants ( $12.3 \pm 0.92$  shoots per culture;  $3.79 \pm 0.28 \text{ cm}$ ; Figure 5 d, Table 1). Similarly, other levels (10–40 mM) showed promotory effect on shoot multiplication, whereas 50 mM putrescine was inhibitory (Tables 1 and 2). Putrescine at 40 mM influenced floral initiation and floral development in both



**Figure 5.** a, Hairy roots of *C. intybus* derived from *A. rhizogenes* LMG-150; b, Shoot regeneration from hairy roots derived from LMG-150; c, Shoot multiplication in untransformed plants of chicory under the influence of 40 mM putrescine; d, Shoot multiplication in transformed plants of chicory under the influence of 40 mM putrescine; e, *In vitro* flowering in untransformed plants of chicory under the influence of 40 mM putrescine; f, *In vitro* flowering in transformed plants of chicory under the influence of 40 mM putrescine; g, *In vitro* seed setting in untransformed (I) and transformed (II) shoot cultures of chicory; h, Germination of *in vitro* obtained seeds from untransformed (I) and transformed (II) shoot cultures of chicory.

untransformed shoot cultures ( $4.6 \pm 0.34$  flowers per shoot apex; Figure 5 e) and transformed shoot cultures ( $1.3 \pm 0.075$  flower per shoot apex; Figure 4 f, Tables 1 and 2), other treatments did not induce *in vitro* flowering.

Of the various treatments with  $\text{AgNO}_3$  (10–50  $\mu\text{M}$ ), 40  $\mu\text{M}$  level in untransformed plants resulted in maximum shoot multiplication ( $36.9 \pm 2.63$  shoots per culture) as well as growth of shoots ( $7.82 \pm 0.76$  cm) compared to the transformed plants ( $11.6 \pm 0.89$  shoots per culture;  $3.2 \pm 0.24$  cm; Tables 3 and 4).  $\text{AgNO}_3$  at 40  $\mu\text{M}$  level also induced floral initiation and floral development in untransformed ( $5.2 \pm 0.39$  flowers per shoot apex) and transformed plants ( $1.4 \pm 0.10$  flower per shoot apex; Tables 3 and 4); other treatments failed to elicit floral response (Tables 3 and 4).

*A. rhizogenes* has been used to obtain transformed and transgenic plants in several systems<sup>12–15</sup>. Hairy roots (LMG-150) and the transformed plants (T1 and T2) regenerated from them showed the presence of *rolA* gene (Figure 1) on PCR amplification, which was absent in untransformed shoots. This was further confirmed by the amplified positive control (plasmid DNA from LMG-150), which led to the establishment of transformed nature of the tissues (Figure 1).

This system has continued to remain stable for over 18 months. The addition of putrescine (40 mM) and  $\text{AgNO}_3$  (40  $\mu\text{M}$ ) resulted in increased shoot proliferation and *in vitro* flowering. We have related the hampered morphogenesis in transformed plants to its altered polyamine metabolism<sup>6,16</sup>. A similar effect of  $\text{AgNO}_3$  on *in vitro*

shoot multiplication<sup>11</sup> and floral bud differentiation<sup>17</sup> has been reported. Other workers reported shoot organogenesis in carrot<sup>18</sup>, alfalfa<sup>19</sup> and tobacco<sup>20</sup>, upon administration of  $\text{AgNO}_3$ . Furthermore, treatments of putrescine in several systems have promoted shoot multiplication<sup>21</sup>.

In our earlier study<sup>16</sup>, we have shown that the altered polyamine metabolism in transformed *C. intybus* plants resulted in altered morphology of the regenerated plants as observed in the form of condensed shoot clusters and short internodes (Figure 5 d). A similar observation was made in tobacco by Tanguy *et al.*<sup>21</sup>. We have also shown that the exogenous polyamines have a physiological role to play in growth and production of secondary metabolites in hairy root cultures of *Beta vulgaris* and *Tagetes patula*<sup>22</sup>. The promotive role of putrescine (40 mM) and  $\text{AgNO}_3$  (40  $\mu\text{M}$ ) was confirmed by the use of polyamines inhibitors<sup>6,16</sup>. It was observed that the transgenic plants of *C. intybus* obtained by *A. rhizogenes*-mediated transformation had shown altered phenotypic character in terms of varying inulin content and changed amino acid profiles, compared to normal plants of chicory<sup>23</sup>. Limami *et al.*<sup>23</sup> found that arginine content in transgenic plants of chicory was directly proportional to change in phenotype. They found that the transgenic plants of chicory behaved as annuals compared to normal biennial nature of *Cichorium* plants. Early flowering of *Cichorium* transformants by insertion of Ri-T-DNA<sup>23</sup> is in contrast to retarded flowering in tobacco proportional to inhibition of the accumulation of polyamines and their hydroxycinnamic acid conjugates<sup>23</sup>.

**Table 1.** Effect of exogenously fed putrescine on phenotypical response in untransformed plants of *C. intybus* L. cv. 'Lucknow local' (values are mean  $\pm$  SD,  $n = 5$ )

Medium + Putrescine (mM)	Number of shoots	Length of shoots (cm)	Number of flowers (per shoot apex)
Control	$19.2 \pm 1.44$	$5.65 \pm 0.42$	—
10	$21.8 \pm 1.63$	$5.2 \pm 0.36$	—
20	$22.6 \pm 1.69$	$6.4 \pm 0.48$	—
30	$25.8 \pm 1.93$	$6.9 \pm 0.51$	—
40	$34.8 \pm 2.61$	$7.69 \pm 0.57$	$4.6 \pm 0.34$
50	$16.4 \pm 1.23$	$2.69 \pm 0.25$	—

**Table 2.** Effect of exogenously fed putrescine on phenotypical response in transformed plants of *C. intybus* L. cv. 'Lucknow local' (values are mean  $\pm$  SD,  $n = 5$ )

Medium + Putrescine (mM)	Number of shoots	Length of shoots (cm)	Number of flowers (per shoot apex)
Control	$6.4 \pm 0.48$	$1.88 \pm 0.14$	—
10	$7.5 \pm 0.56$	$1.86 \pm 0.13$	—
20	$8.2 \pm 0.61$	$2.3 \pm 0.17$	—
30	$9.6 \pm 0.72$	$2.32 \pm 0.28$	—
40	$12.30 \pm 0.92$	$3.79 \pm 0.28$	$1.3 \pm 0.075$
50	$5.06 \pm 0.375$	$0.91 \pm 0.06$	—

**Table 3.** Effect of exogenously fed  $\text{AgNO}_3$  on phenotypical response in untransformed plants of *C. intybus* L. cv. 'Lucknow local' (values are mean  $\pm$  SD,  $n = 5$ )

Medium + $\text{AgNO}_3$ ( $\mu\text{M}$ )	Number of shoots	Length of shoots (cm)	Number of flowers (per shoot apex)
Control	$19.2 \pm 1.44$	$5.65 \pm 0.42$	—
10	$22.6 \pm 1.69$	$5.6 \pm 0.36$	—
20	$24.2 \pm 1.86$	$6.9 \pm 0.49$	—
30	$28.9 \pm 2.01$	$6.96 \pm 0.51$	—
40	$36.9 \pm 2.63$	$7.82 \pm 0.76$	$5.2 \pm 0.39$
50	$15.2 \pm 1.01$	$3.1 \pm 0.21$	—

**Table 4.** Effect of exogenously fed  $\text{AgNO}_3$  on phenotypical response in transformed plants of *C. intybus* L. cv. 'Lucknow local' (values are mean  $\pm$  SD,  $n = 5$ )

Medium + $\text{AgNO}_3$ ( $\mu\text{M}$ )	Number of shoots	Length of shoots (cm)	Number of flowers (per shoot apex)
Control	$6.4 \pm 0.48$	$1.88 \pm 0.14$	—
10	$7.1 \pm 0.51$	$1.73 \pm 0.09$	—
20	$7.5 \pm 0.59$	$2.13 \pm 0.15$	—
30	$8.6 \pm 0.62$	$2.3 \pm 0.17$	—
40	$11.6 \pm 0.89$	$3.2 \pm 0.24$	$1.4 \pm 0.10$
50	$5.4 \pm 0.41$	$0.91 \pm 0.06$	—

Chicory is being used as vegetable, coffee blend, metabolite of medicinal and cosmetic value, viz. esculin and esculetin<sup>24,25</sup> and as hepatoprotective agents<sup>26</sup>. This has led to large-scale cultivation of this crop and there is interest to genetically engineer them to obtain higher yields and for newer potentials. Male sterile lines of chicory have been obtained by incorporating barnase gene<sup>27</sup>.

The present study led to floral induction in chicory plants. These plants, which would otherwise flower biennially, can be induced to flower precociously for studies on *in vitro* pollination and seed development (Figure 5 g). *In vitro* obtained seeds from both transformed and untransformed plants were found to be viable on testing them for their germination efficiencies on MS basal medium (Figure 5 h).

This crop being amenable to genetic manipulation would be the crop of choice for genetic manipulation. The information already generated in terms of available transformation protocols, *in vitro* morphogenesis, including flowering and seed set will make the target of genetic transformation for crop improvement. In future this approach would enhance the potential of this crop for economic benefits.

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## Geomorphological evidences of retreat of the Gangotri glacier and its characteristics

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**The Gangotri glacier which has the 258.56 sq km of glacierized area is receding as evidenced by various geomorphological features and morphometry parametric. Because of subsidence and the fast degenerating nature of the glacier, middle part of ablation zone is full of supraglacial lakes. The study shows that retreat was much slower before, compared to what was after 1971. Series of hummocky moraines indicate a faster retreat of the ice.**

THE Gangotri glacier is one of the largest Himalayan glaciers, which is about 30.20 km long<sup>1</sup> with its width varying from 0.5 to 2.5 km. It is a valley-type glacier, situated in the Uttarkashi district of Garhwal Himalaya, Uttaranchal (Figure 1) and it flows to NW direction. This glacier is bound between 30°43'22"–30°55'49" (lat.) and 79°4'41"–79°16'34" (long.), extending in height from 4120 to 7000 m.a.s.l. This area is situated north of the Main Central Thrust (MCT) and comprises bed rocks of

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