Indian Journal of Experimental Biology
Vol. 60, February 2022, pp. 121-130

Development of transgenic cucumber mosaic virus (CMV) resistant gerbera plants expressing CMV coat protein gene

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Received 27 May 2020; *revised 18 January 2022*

Gerbera (*Gerbera jamesonii* L.) has its immense importance to the floriculture industry worldwide. The gerbera flower production has been hampered by various viruses, among them cucumber mosaic virus (CMV) has shown considerable damage.As natural resistance to CMV is absent in gerbera, here, we have made an attempt to develop transgenic gerbera plants expressing coat protein (*CP*) gene of CMV via *Agrobacterium* mediated transformation of base petiole explants for genetic resistance to CMV infection. Among the 44 putative transgenic gerbera plant acclimatized, 39 were found positive for integration of *CP* gene by polymerase chain reaction and southern hybridization assay using their specific primer and probe respectively. Northern hybridization assay using CP gene specific probe confirmed the transcription of transgene in all 39 transgenic plants. These plants showed translation of CP during DAS-ELISA when tested with antiserum specific to CP of CMV. These 39 plants when challenged by mechanical inoculations with CMV gerbera isolate showed virus resistance in 53% (21 out of 39) plants, virus tolerance (delayed mild symptom) in 33% (13/39) plants, while rest 12.8% (5/39) plants showed severe disease symptoms. The CP mediated resistance of CMV in transgenic gerbera is being reported for the first time from India.

Keywords: Challenge inoculations, Floriculture industry, Ornamental crops, RNA virus, Virus resistance

Gerbera jamesonii L. is a popular ornamental plant grown in beds or pots, and used as cut-flower in bouquet and decoration during ceremonial functions. It is commercially cultivated by a large number of growers in India as a primary source of income, therefore, has high socioeconomic impact in floriculture industry. In India, gerbera cultivation accounts for about 800 hectares in floricultural sector and produce about 25.56 tonnes of lose and cut flower gerbera during 2015-16 and ranked 29 in global position (http://apeda.in/agriexchange/India%20Production/India_Productions.aspx?hscode=1030). Trait improvement in gerbera is focused on the production of high valued quality blooms, however availability of the disease-free planting material is limited by occurrence of pathogens[1]. The cultivation of gerbera is seriously limited by the natural occurrence of many viruses including impatiens necrotic spot virus (INSV)[2], tomato spotted wilt virus (TSWV)[3-5], tobacco rattle virus (TRV)[6,7], tomato black ring virus (TBRV) and tobacco mosaic virus (TMV)[8], and cucumber mosaic virus (CMV)[9-12]. Virus diseases in gerbera cannot be managed post infection, and therefore warrant non-conventional management approaches. Literature survey reveals only few reports of natural occurrence of CMV on gerbera all over the world[9-12]. The natural occurrence of CMV on gerbera exhibiting colour break symptoms on petals, asymmetrical ray florets, and deformed flowers was reported for the first time in India by Verma and co-workers[12]. Later, an isolate of CMV of subgroup IB causing severe chlorotic mosaic and flower deformation in gerbera plants was earlier identified by sequence analysis of complete RNA 3 genome by our group[10,11].

CMV is considered as a bottleneck of the crop production because it caused significant losses in most of the major crops around the world[13,14], therefore, protection against CMV seems to be essential by generating virus resistance/tolerance in crop plants. In this context, Cuozzo and co-workers in

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[*Abbreviations*: CMV, cucumber mosaic virus; CP, coat protein; CPMR, coat protein mediated resistant; DAC-ELISA, direct antigen coating enzyme linked immunosorbent assay; MS, Murashige and Skoog's; IAA, Indole-3-acetic acid; BAP, 6-Benzyl aminopurine; AdSo4, adenine sulphate; and TDZ, Thidiazuron]

1988 demonstrated the coat protein-mediated resistant (CPMR) against the CMV for the first time in tobacco[15]. Then after, CPMR has shown varying degrees of resistance with different constructs in different host plants. In India, CPMR was used to generate resistance against CMV subgroup IB isolate in transgenic lines of *Nicotiana benthamiana*[16]; chrysanthemum[17] and tomato[18]. The resistance against CMV subgroup IA isolate was also demonstrated in *N. tabacum*[19]. Development of the transgenics for different traits like morphological changes, longer stability or creating resistance against viral diseases has considerable potential in ornamental crops[20-22]. Gerbera flower crop is commercially cultivated by farmers as a primary source of income. In the present study, we have made an attempt to develop transgenic plants of gebera cv. Zingaro, using leaf, petople and base petole explants, expressing the *CP* gene of an Indian isolate of CMV of subgroup IB[16]. The resistance to a CMV isolate of gerbera in transgenic plants has also been demonstrated.

Materials and Methods

Explant source

The floral pedicles excised from healthy plants of *Gerbera jamesonii* cv. Zingaro were surface sterilized with 0.1% (w/v) mercuric chloride solution followed by six washes in sterile distilled water and blot dried. These explants were used to aseptically regenerate direct shoots on MS medium and were propagated in half strength MS medium[23]. The aseptically regenerated plants were used as source of leaf, petiole and base petiole explants during further experiments. For standardization of transformation protocol, explants each of leaf lamina (10 mm^{-2}), petiole (7 mm) and base petiole (5 mm) were screened for optimum response in MS medium supplemented with combinations of indole-3-acetic acid (IAA), 6-benzylaminopurine (BAP), zeatin (Zn), thidiazuron (TDZ), and adenine sulphate (AdSo4) growth regulators (Table 1). All cultures were maintained at 25±2 ·C temperature with 16 h photoperiod (50 mol/m2/s quantum flux density) at culture level as suggested[24].

CMV-CP construct and *Agrobacterium* strain used for gerbera transformation

CP gene of CMV introduced into a binary vector p*RoK*2 (Suppl. Fig S1. *All supplementary data are available only online along with the respective paper at NOPR repository at http://nopr.res.in*) and transformed by tri-parental mating in *Agrobacterium tumefaciens* strain LBA4404 earlier by our group was used for this study[16]. Restriction digestion analysis of plasmid DNA isolated using QIAprep Spin Miniprep Kit (QIAGEN India Pvt. Ltd.) from the clones in *A. tumefaciens* was done with *Xba*I and *Kpn*I restriction enzymes for confirming the presence of *CP* gene in them. The positive clones were further verified by PCR using *CP* gene specific primers and then used for transformation of gerbera explants. For PCR, total plasmid DNA isolated was used as template for amplification of the *CP* gene of 650 bp length using *CP* gene specific primer pair (Accession numbers: AM180922/AM180923). The CMV-CP cloned DNA in p*RoK*2-*Agrobacterium* was used as positive control. PCR was done following the protocol of Kumar and co-workers[17] in a 25 µL reaction volume. The initial denaturation was at 95°C for 30s following 30 cycles of denaturation at 95 ·C for 30 s, annealing at 55 ·C for 30s and extension at 72 ·C for 60s with a final extension cycle of 7 min at 72 ·C. PCR products were separated by gel electrophoresis in 1% agarose with Lambda DNA digested with *EcoR*I/*Hind*III as DNA markers.

Development of transgenic plants

Agrobacterium mediated transformation of base petiole explants of gerbera cv. Zingaro was performed following the procedure of Eloma *et al*.[25] with slight modifications. Fresh base petiole explants were excised from the 3-4 upper leaves of approximately 5 mm in size from aseptically grown gerbera plants. Explants were blot dried and placed on MSc1-C callus inducing medium (basal MS medium 1.0 mg/L BAP, 0.5 mg/L IAA, 1.0 mg/L Zeatin, 3% glucose and 0.8% agar) (Table 1) and incubated at 25± 2°C in the culture room at 16 h light/ 8 h dark day cycle for 2

Table 1 — Standardization of callusing, shoots proliferation, and elongation media

Plant hormones	Media											
	MSc1				MSc2				MSp			
	A	B	C	D	A	B	C	D	A	B	C	D
IAA (mg/L)	0.1	0.1	0.5	0.25	0.1	0.1	0.1	0.25	0.1	0.1	0.1	0.1
BAP (mg/L)	0.5	1.0	1.0	1.0	0.5	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Ads (mg/L)	0.5	1.0	1.0	1.5	0.5	1.0	1.0	1.5	2.0	1.0	0.5	0.25
Performance of explants	+	+	+++	++	+	+	+++	++	+	++	+++	++

[+++ = most suitable, ++ = good/moderated and + = poor]

days. Co-cultivation with *Agrobacterium* culture carrying CP-construct was done and explants were incubated on the same medium for 48 h at 25±2°C under a dark condition in the culture room for callus induction. After two days of co-cultivation with *Agrobacterium*, the explants were transferred on to MSc2-C-S callus development medium (1.0 mg/L BAP, 0.1 mg/L IAA and 1.0 mg/L Zeatin) supplemented with 25 mg/L Kanamycin and 250 mg/L Cefotaxime for selection and callusing (Table 1). The explants were transferred to fresh selection plates at every 14-15 day interval for 2 to 3 successive growth cycles (21-30 days). These calli were transferred on to MSp-C shoot development medium (1.0 mg/L BAP, 0.1 mg/L IAA and 0.5 mg/L AdSo4) (Table. 1) for shoot proliferation and elongation.

Rooting and acclimatization of in vitro regenerated gerbera plants

Well organized shoots (with 4-5 leaves and an apical meristem from which any developed callus was removed) were transplanted in MSr rooting medium (MS basal supplemented with 0.1 mg/l IBA). Once a branched root system developed, the plants were released from the medium, agar was thoroughly washed off from the roots and were transferred in small pots containing vermiculite. Pots were kept in culture room for 2 weeks for acclimatization and hardening. These developed transgenic plants were finally planted and grown in 12 inches earthen pots containing soil:sand:vermicompost in 1:1:2 ratio in glasshouse.

Confirmation of CP integration in putative transgenic plants by PCR and Southern hybridization

To check the integration of *CP* gene in transgenic gerbera plants, the total genomic DNA was isolated[17] from transgenic and untransformed healthy plants (as negative control). The isolated DNA was used as template for amplification of the *CP* transgene in PCR using *CP* gene specific primers. The CMV-CP cloned DNA in p*RoK*2-*Agrobacterium* was used as positive control. PCR products along with Lambda DNA digested with *Eco*RI/*Hind*III as size marker were separated on 1% agarose gel by electrophoresis to check the amplification of *CP* gene. To confirm the integrity of the amplified products, Southern hybridization assay was performed using a homologous probe, prepared from a positive clone of CMV-CP gene labeled with radioactive αP32 dCTP by random primer labeling method[26]. The steps of Southern hybridization were followed as suggested earlier[17].

Northern hybridization assay for confirmation of transcription of CP gene in transgenic plants

Further, the RNA transcripts of *CP* gene in transgenic gerberas were assessed by Northern hybridization assays. Briefly, total genomic RNA was isolated from 1.0 g leaf tissue of transgenic plants employing RNA isolation kit (Sigma-Aldrich, USA) as per the manufacturer's instructions. The isolated RNA from CMV infected and healthy uninoculated untransformed plants were used as positive and negative references. A total of 5.0 µg RNA from each sample was blotted and cross-linked on Hybond N+ nylon membrane (Sigma-Aldrich, USA). The probe prepared from about 350 bp sequence at 3′ end of the *CP* gene by random primer labeling method[26] was deployed for hybridization of blotted DNA bands. Prehybridization and hybridization were carried out at 42°C with 50% formamide and blots were washed as instructed. Further, the isolated RNA from inoculated and systemic leaves of selected progeny plants was subjected to Northern hybridization assay to determine the level of RNA accumulated after CMV challenge inoculations.

Western immunoblot assay of transgenic plants

To observe CP production in transgenic plants, 20 µg total plant proteins were separated by SDS PAGE using 12% polyacrylamide gel. The resolved protein bands were transferred on Immun-Blot PVDF Membrane (Bio-Rad) in a minitransblot apparatus (Bio-Rad) and expression of protein level was determined using CMV specific antiserum (PVAS 242a, ATCC, USA).

DAS-ELISA based assessment of transgenic plants after challenge inoculations of CMV

Transgenic plants were assessed by mechanical challenge inoculations for resistance against a CMV isolate of gerbera cv. Zingaro plants[10]. For this, one month old transgenic and healthy untransformed control plants were mechanically inoculated on their 3rd leaf with the leaf sap of CMV infected gerbera plant. The inoculums were prepared in 0.1 M sodium phosphate buffer (pH 7.0) and 1% (w/v) sodium sulphite and inoculated on third young leaf of gerbera plants using fine carborundum abrasive (Silicon carbide) and observed up to 45 days post-inoculation (dpi) for symptom development as compared to uninoculated untransformed plants. Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) was performed to assess the relative level of CP accumulation in upper 5th uninoculated

leaf of transgenic lines after CMV inoculations corresponding to the untransformed diseased control plant. The leaf sap from the test plants was extracted, diluted to 1:50 ratio in PBS buffer and used in DAS-ELISA following the protocol as shown in Srivastava and Raj[16]. The CMV specific PVAS242a antiserum at 1:500 dilution and alkaline phosphates conjugate at 1:1000 dilution were used as primary and secondary antibody, respectively for tagging the CP. All the experiments were done in triplicate. The CP level in transgenic plants was determined by comparing the absorbance from CMV inoculated untransformed positive control plant. The total protein from healthy untransformed gerbera plants was used as negative reference control for all DAS-ELISA experiments.

Results

Optimization of transformation parameters

The standardization of transformation protocol was a prerequisite for the development of transgenic gerbera plants. The leaf, petiole and base petiole explants from healthy gerbera plants were used as source of explant (Fig. 1) for optimization of regeneration protocol using different growth hormone combinations (Table 1). Results showed that the base petiole explants responded best in all combinations and efficient rate of regeneration was achieved. The petiole explant regenerated by comparatively at a low frequency whereas leaf explants showed poorest response and were hard to regenerated on this medium (Fig. 1).

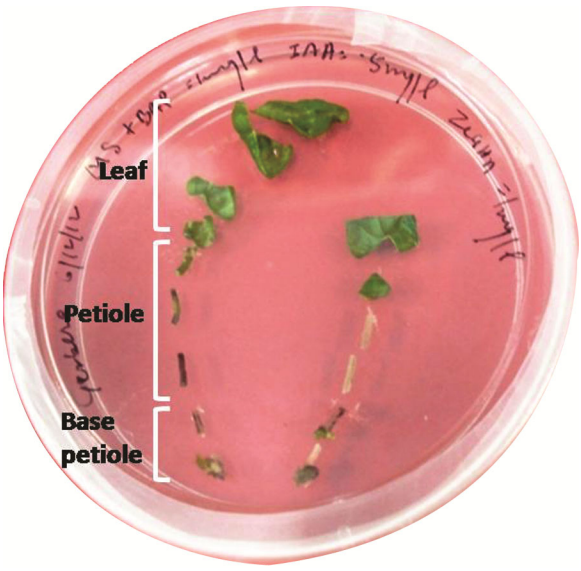

Fig. 1 — Gerbera leaf, petiole, and base petiole explants taken at 15 days on MSc-1 callusing medium showing comparative callusing among them

Amongst the various combinations of plant growth hormones selected, the MS basal medium supplemented with 0.5 mg/L IAA, 1.0 mg/L BAP and 1.0 mg/L Zeatin was found best for callus induction and termed as MSc1-C (Table 1). Later on, callus was efficiently developed MS medium supplemented with 0.1 mg/L IAA, 1.0 mg/L BAP and 1.0 mg/L Zeatin and termed as MSc2-C (Table 1). For shoot proliferation and elongation, MS medium supplemented with 0.1 mg/L IAA, 1.0 mg/L BAP and 0.5 mg/L AdSO4 was used, termed as MSp-C (Table 1). The multiple shoots were produced from base petiole explants then transferred to the rooting medium supplemented with 0.1 mg/L IBA, termed as MSr. Shoot regenerated from the base petiole explants were used for gerbera transformation. The established protocol was found reproducible and simple for raising transgenic plants.

Transformations of gerbera with CMV-CP gene construct using base petiole explants

A. tumefaciens mediated transformation of base petiole explants of gerbera cv. Zingaro was carried out following the established protocol[25]. A total of 310 base petiole explants obtained from aseptically grown gerbera plants were transformed with CMV-CP gene construct (Suppl. Fig. S1) in different events following the aforesaid optimized conditions. Out of which, only 97 explants (31.29% of the total) (Table 2) survived on MSc2-C-S medium supplemented with 25 mg/L Kanamycin and 250 mg/L Cefotaxime and showed sign of green callus regeneration (Fig. 2A). After 8-10 weeks of co-cultivation, little green calli formed at the end of petiole. At this stage, the untransformed explants turned completely brown and died on the selection medium. The green calli were removed and transferred to fresh MSc2-C-S medium. After three weeks subcultures, few shoots were originate from green calli (Fig. 2B) which were excised from callus and transferred to MSp-C medium for proliferation and elongation. After two weeks, the shoots of average height of 3-4 cm (Fig. 2C) were sub cultured in MSr rooting medium (Fig. 2D). Hairy root formation was initiated at the base of shoot nearly after two weeks of incubation in rooting medium which were developed completely in one month time (Fig. 2D). The well rooted 50 (51.54%) gerbera plants were transferred in vermiculite containing pots (Fig. 2E) and kept in acclimatization chambers for a week in culture room conditions. The 44 (88%)

Table 2 — *Agrobacterium* mediated transformation, regeneration and screening of putative transgenic plants of gerbera cv. Zingaro

Events	Total explants employed	Explants survived on (MSc2-C+S)* medium and developed shoots	No. of shoots rooted in MSr medium	Rooted plants survived after hardening in glasshouse
A	140	41	22	19
B	100	35	17	16
C	70	21	11	9
Total	310	97 (31.29%)	50 (51.54%)	44 (88.0%)

[*MSc2 = Basal MS + Hormones (BAP = 1.0 mg/L + IAA = 0.1 mg/L + Zeatin = 1.0 mg/L), S = (Kanamycin = 25 mg/L, Cefotaxime = 250 mg/L) and MS2 = Basal MS + IAA = 0.1 mg/L). Finally, 44 well putative transgenic gerbera plants established in glasshouse and were validated for presence and transcription of CP gene]

Fig. 2 — Successive regeneration stages of transformed base petiole explants of gerbera cv. *Zingaro*. (A) Explants survived on MS1-C-S medium after 15 days; (B) survived explants initiated shoot formation after 30 days on MS2-C-S medium; (C) proliferation of 2-4 shoots after 60 days on MSp-C; (D) rooting in 21-25 days in gerbera rooting medium; (E-F) acclimatization of plants in glasshouse conditions; and (G) gerbera plant at maturity and blooming stages in glasshouse

survived gerbera plants in culture room were settled in glasshouse condition for 10-12 days. After that, these plants were transferred in potting mixture (mature leaf compost: top soil: sand in 2:1:1 ratio) for proper growth and flowering (Fig. 2 F and G).

Validation of putative transgenic plants by PCR, Southern and Northern hybridization assays

For validation of the transgenic plants, total genomic DNA and RNA was isolated from the leaf samples and analyzed at the molecular level for the successful integration and transcription of the CMV-CP transgene in gerbera using PCR, Southern hybridization and northern blot analyses. The 44 acclimatized plants of putative transgenic gerbera were tested by PCR using CMV-CP gene specific primers. cDNA from an infected gerbera plant taken as positive control. PCR was found to be positive in 39 (88.6%) plants that showed expected size ~650 bp amplicon similar to the positive control. Five plants were found to be negative for CP transgene as no positive amplicon was observed during PCR. A representative gel of eight samples selected randomly, along with positive and negative control has been shown in (Fig. 3A). The obtained PCR results were further confirmed by Southern blot analysis using CP gene probe which resulted in

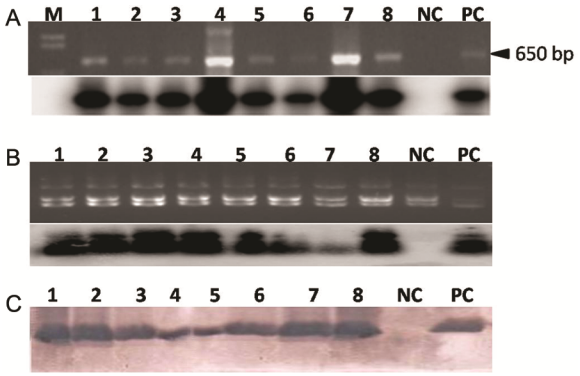

Fig. 3 — Representative gel image (A) showing expected size ~650 bp DNA band in 8 positive transgenic plants of gerbera (Lanes 1-8) in PCR; (B) amplicons showing positive signal of hybridization in Southern hybridization assay deploying CMV-CP gene specific probe; and (C) Northern hybridization assay of total genomic RNA of all transgenic plants using the same probe. [M = Standard DNA as size marker, NC = Negative control, PC = positive control]

positive signals of hybridization in all the PCR positive transformants (Fig. 3A). To analyze the transcription of CP gene in putative transgenic plants, Northern hybridization tests were performed using total RNA isolated from these plants. The RNA of CMV-infected and healthy untransformed gerbera plants were also taken as positive and negative control. All the RNA samples were blotted on nylon membrane and allowed to hybridize with CMV-CP specific probe (prepared from a CMV-A clone). The putative transgenic plants and positive control samples showed strong signals on the developed blot whereas the untransformed healthy gerbera plants did not showed any signal of hybridization (Fig. 3B).

Confirmation of translation of CP by and Western immunoblot assays and DAS-ELISA in transgenic plants

Western blot immunoassay using antiserum to CMV (PVAS 242a) also detected a protein of about 24 kDa in the total leaf protein isolated from assayed plants (Fig. 3C), confirming the *in vivo* translation of CP mRNA in the leaves of these selected transgenic plants. These results confirmed the successful integration and transcription of the CP gene in all 39 transgenic plants.

For assessment of CP level in transgenic gerbera plants, the ELISA reading (absorbance at 450 nm wavelength) of transgenic plants were recorded and compared with negative control (healthy) and positive control (CMV inoculated) plants. The absorbance data of all the test plants obtained in triplicate has been summarized in table 3 which showed presence of CP protein in all the 39 transgenic plants tested by DAS-ELISA using CMV specific primary antibody (antiserum PVAS 242a). The CP accumulation level in CMV inoculated positive control gerbera plants was highest as the absorbance was obtained as more than 1.30. The high accumulation of CP protein was observed in 48.7% (19/39) transgenic gerbera plants (as absorbance obtained was 1.15-1.28) which were almost similar to positive control (Table 3). The moderate CP accumulation (1.00-1.14) was also

Table 3 — Absorbance obtained in ELISA reader at 450 nm from transgenic gerbera plants as compared to positive (PC) and negative (NC) control

Sample	Absorbance*	Standard error
Buffer	0.177	0.034
Negative control	0.786	0.162
Positive control	1.378	0.075
T-1	1.117	0.080
T-2	1.168	0.031
T-5	1.284	0.101
T-4	1.176	0.233
T-5	1.231	0.325
T-6	1.156	0.357
T-7	1.087	0.236
T-8	1.052	0.372
T-9	1.158	0.186
T-10	1.169	0.314
T-11	1.189	0.287
T-12	1.131	0.136
T-13	1.119	0.273
T-14	1.177	0.136
T-15	1.194	0.183
T-16	1.185	0.202
T-17	1.135	0.094
T-18	0.814	0.064
T-19	1.194	0.215
T-20	1.068	0.070
T-21	1.117	0.249
T-22	0.837	0.085
T-23	1.157	0.236
T-24	1.178	0.345
T-25	1.172	0.218
T-26	1.113	0.113
T-27	1.186	0.361
T-28	1.133	0.054
T-29	0.826	0.162
T-30	1.146	0.183
T-31	1.212	0.252
T-32	0.828	0.078
T-33	0.823	0.066
T-34	1.270	0.274
T-35	1.127	0.336
T-36	1.136	0.315
T-37	1.109	0.243
T-38	1.143	0.187
T-39	1.159	0.139

[*Absorbance (average value of a triplicate experiment) considered in transgenic plants as High for A1.15 to A1.28, moderate when A1.0 to 1.14 and low when A0.81 to 0.83]

observed in 38.4% (15/39) transgenic plants. However, the accumulation of CP was low (0.81-0.83) in 12.8% (5/39) transgenic plants (T18, T22, T29, T32 and T33) (Table 3). These results indicated successful translation of CMV-CP gene in about 87% transgenic gerbera plants developed out of the study.

Validation of transgenic gerbera plant by challenging with CMV

All the 39 transgenic gerbera plants, ascertained by different assays, were then challenged by a CMV isolate of gerbera through mechanical inoculations. The untransformed uninoculated healthy and untransformed CMV infected gerbera plants served as reference control. The high accumulation of CP was observed in 48.7% (19 out of 39) transgenic plants (T-2, T-3, T-4, T-5, T-6, T-9, T-10, T-11, T-14, T-15, T-16, T-19, T-23, T-24, T-25, T-27, T-31, T-34 and T-39) as absorbance (A 405 nm) observed ranged from 1.39 to 2.14, which was somewhat similar to the positive control (2.27 at A 405 nm) after 21 dpi (Fig. 4). These gerbera plants showed varying degree of mild to severe mosaic symptoms on leaves at 21 dpi. The moderate CP accumulation from 0.90 to 1.27 at A 405 nm was observed in 38.4% (15 out of 39) transgenic plants (T-1, T-7, T-8, T-12, T-13, T-17, T-20, T-21, T-26, T-28, T-30, T35, T-36, T-37, and T-38) after 21 dpi. The symptoms observed in these transgenic plants vary from asymptomatic to mild mosaic on leaves. Least accumulation of CP ranging from 0.41 to 0.63 at A 405 nm was observed in 12.8% (5 out of 39) transgenic plants (T18, T22, T29, T32, and T33) at 21 dpi. These plants displayed very mild mosaic to no symptom when observed at 21 dpi. Challenge inoculation study revealed that out of 39 challenged transgenics, 5 (12.8%) plants were found to be resistant to CMV since they remained asymptomatic throughout their life and bloomed like healthy uninoculated untransformed plants. While, 15 plants (38.4%) were found tolerant which showed delayed and variable degree of mild symptoms of virus infection, even at 45 dpi. Such plants showed comparatively smaller flower with colour distortion symptoms. Contrary to this, the 19 transgenic plants (48.7%) that accumulate high CP (1.39 to 2.14 at A 405 nm), which was corresponding to the CMV infected untransformed positive control plant, were failed to provide protection against the CMV and displayed severe yellow chlorotic symptoms at 45 dpi. The flowers in these plants were comparatively small with discoloured and deformed ray florets. A clear variation in size and colour of ray florets was observed in these plants when representative flowers were compared (Fig. 5). These results indicated the developed of resistance and/or tolerance in transgenic gerbera plants against the CMV infection.

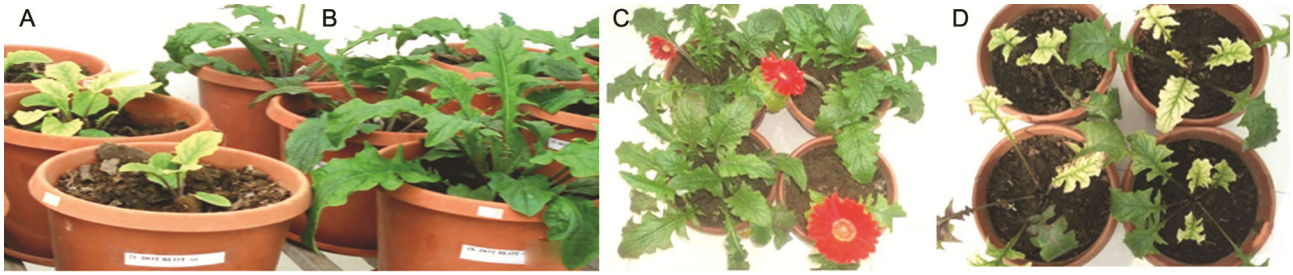

Fig. 4 — Challenged gerbera plants with CMV showing (A) severe chlorotic and yellowing in non-transgenic plants; (B) while no symptoms in challenged transgenic plants at 45 dpi; Challenged transgenic gerbera plants showing (C) full blooming in glasshouse conditions; as compared to (D) un inoculated non transgenic plants

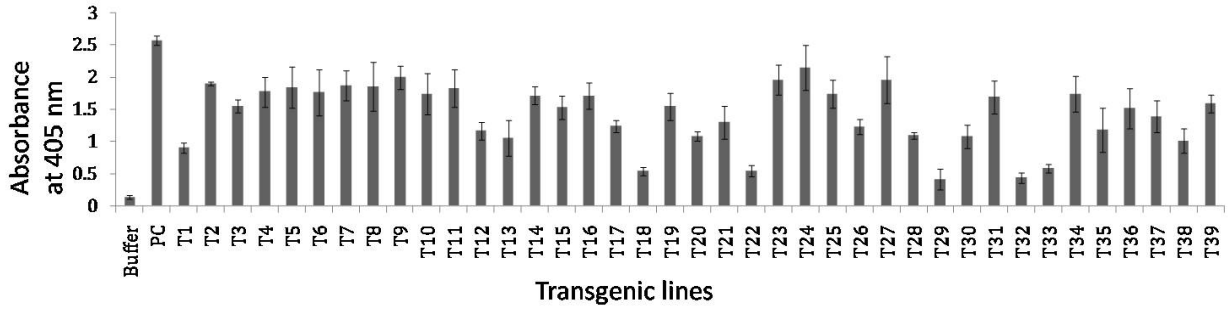

Fig. 5 — DAS-ELISA based screening of all mechanically inoculated 39 transgenic gerbera plants using CMV specific PVAS 242a antiserum. The data was generated in triplicate with 1:50 dilutions of crude sap of leaf from transgenic plants. Bar represent the standard error

Discussion

The CMV has shown high important because it causes severe flower deformations in gerbera and comparatively more damage to the marketable value of its blooms[10], therefore, management of CMV has become essential. Keeping in view of the trait improvement and development of in-built resistance against viruses in gerbera, the genetic transformation protocol using CMV-CP gene cassette[16] and leaf, petiole and petiole base explants of gerbera was optimized in present study. When different hormones combination were added to basal MS medium, base petiole of explant has shown higher regeneration efficiency as compared to the petiole and leaf explants. The earlier references on use of Kanamycin were between 7 to 25 mg/L and cefortaxime concentration were 250-500 mg/L for gerbera transformation[25,27,28]. For the present study, use of 25 mg/L concentration of Kanamycin and 500 mg/L concentration of cefortaxime was found optimum during the *Agrobacterium* mediated transformation experiments of base petiole explants of gerbera. A total of 310 base petiole explants of gerbera cv. Zingaro were co-cultivated for transformation and out of them 97 were successfully regenerated on MSc1-S selection medium. As high as 4-5 shoots were regenerated from a callus. All shoots were transferred to MSr rooting medium where 52% (50) plants had developed a well organized branched root system. The developed transgenic plants were placed under greenhouse conditions for acclimatization from where the survived 44 (88%) plants were chosen for molecular validation. The results of PCR, Southern and Northern blot analysis confirmed that total 89% (39) plants had CP gene integration in their genome.

The transgenic plants when challenged with leaf sap inoculums of CMV, 53% (21) plants did not show any symptoms of CMV infection while 33% (13) plants showed mild symptoms that were delayed by 15 days. Almost similar results have been reported during transformation of different ornamental or vegetable crops[17,18,29]. In gerbera crop, the development of transgenic plants expressing N-gene TSWV was achieved earlier[27] and it is the only study for developing the genetic resistance against virus in gerbera. They had used shoots, bases of shoot clumps, and leaves with 2-3 mm lamina length as explant source for transformation of N-gene of TSWV in pBin19 plasmid by *A. tumefaciens* (strain LBA4404) and developed 74 transgenic gerbera plants with 5.4% transformation efficiency. The resistance to TSWV in transformants was demonstrated even with the low level of virus nucleoprotein expression. Our study is the first demonstration of CP mediated protection of gerbera plants against the naturally occurring CMV gerbera isolate of subgroup IB. By and large efforts, the 12.5% transformation efficiency has been achieved with the optimized system. Moreover, protection in the form of resistance and/or tolerance (delayed symptoms) against the CMV has been achieved in about 87.1% developed transgenic gerbera plants. The resistant gerbera progeny failed to develop any symptom throughout their life and produce quality bloom which is in consonance with the finding of Korbin *et al*.[27]. However the actual resistance mechanism is solely either through CP or its RNA or due to the CP and RNA is point of debate[30,31] and need investigations. Our findings corroborate well with the findings of Kumar *et al*.[17] where delayed resistance against CMV isolate of chrysanthemum was achieved in transgenic chrysanthemum plants expressing the *CP* gene, and is also a crop propagated by vegetative cuttings.

The overall quality and quantity of production has been decreased specially in ornamental crops due to virus infection in plants. Hence, it is important to focus on virus disease management of these ornamental crops and to create virus-resistant plants. Coat protein-mediated resistance (CPMR) is used to obtain resistance and has emerged as promising and successful approaches against many viruses including CMV. It has shown variable results on resistance in transgenic plants against viruses in different crops, which might be partly due to differently accumulation of CP protein in different host systems[19].

In the previous years, many viral genes including the coat protein (*CP*) gene have been transferred to developed virus resistant plants. The *CMV-CP* gene is used in several plant species, such as tobacco[16,19,32,33], cucumber[34], tomato[18,35,36], melon[37], squash[38], pepper[39] and chrysanthemums[17] . A combination of two *CP* genes, representing both subgroups of CMV was used in tomato plants transformation where the transformed plant showed extremely high resistant to CMV infection[36]. It has been proved that transformation using the CP gene to obtain virus-resistant plants is a successful approach to create resistance against different plant viruses[15]. However, high levels of *CP* expression is needed to obtain operational levels of resistance. Since most of the

floricultural and ornamental crops are non-edible and grown only for aesthetic and decorative purpose, the bio-safety issue is not of that concern as in other food crops.

Conclusion

A highly efficient *Agrobacterium* mediated transformation method was established for gerbera cv. Zingaro using base petiole as explants. The present study is the first record of genetic transformation of Indian gerbera for development of resistance against cucumber mosaic virus (CMV). This protocol may be adopted for transferring any character/genes of agronomic interest in gerbera plants.

Acknowledgement

Authors are thankful to the CSIR, New Delhi for funding as PMSI project number BSC-117. Authors KKG abd RR thank the ICMR and UGC, respectively for providing fellowships.

Conflict of interest

Authors declare no competing interests

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