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IMPACT OF *AGROBACTERIUM TUMEFACIENS* CO-CULTIVATION TIME AND TEMPERATURE ON T-DNA TRANSFER AND EXPRESSION IN PLANT CELLS

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Abstract: Seventeen *Agrobacterium tumefaciens* strains were used to study the effects of co-cultivation time and temperature on the transformation efficiency of these strains in callus suspension cultures and freshly excised callus pieces of *Brassica oleracea* L. Amongst these seven strains, AM1, EI1, EI2, EI3, MI2, MI3 and MI4 were used to check the effects of co-cultivation time and temperature on the transformation efficiency of these strains in explants (leaf discs, stem cuttings and root cuttings) of five different species (*Capsicum annuum* L., *Lycopersicon esculentum* Mill., *Medicago falcata* L., *Raphanus sativus* L. and *Spinacea oleracea* L.). T-DNA transfer and expression was demonstrated by auxin independent growth of calluses/explants. Results of these experiments revealed that time and temperature play an important role in the oncogenicity, virulence and transformation efficiency of the *A. tumefaciens* strains. Generally co-cultivation for a longer period (10 minutes) and at higher temperature (37°C) yielded no transformation response. Albeit the co-cultivated calluses/explants were shifted to 24°C for T-DNA transfer and expression, but co-cultivation at higher temperature caused irreversible damage. Although varied and individual responses were observed with different strains but in general co-cultivation for 3 minutes at 28°C exhibited best transformation response for majority of strains in callus suspensions, callus pieces and explants. Transformation responses were better in callus suspensions and callus co-cultivations relative to explant co-cultivations. Co-cultivation of callus and callus suspension at 24°C for 3 minutes also exhibited transformation response but to a lesser extent. The AM1, EI2 and EI3 showed better and efficient transformation response when co-cultivated for 3 minutes time interval at 28°C with callus, callus suspension and explants. These strains also showed various morphogenetic responses in callus and callus suspension co-cultivations which were not observed with other strains.

Key words: *Agrobacterium tumefaciens*, co-cultivation, time, temperature, transformation efficiency.

INTRODUCTION

Agrobacterium tumefaciens is a soil phytopathogen which causes crown gall disease on a variety of plants (Hooykaas and Schilperoort, 1992; Howard and Citovsky, 1990; Shaw *et al.*, 1991). The virulent strains of *A. tumefaciens* harbor Ti plasmid, which ranges in size from 190 to 250 Kb, that carries genes essential for crown gall tumor induction (Wabiko *et al.*, 1990; Shurvinton and Ream, 1991). The virulence (*vir*) region of Ti plasmid is required for the transfer of T-DNA from *A. tumefaciens* to plant cells where it integrate in plant genome (Hille *et al.*, 1984; Lundquist *et al.*, 1984). Three genes (*tmsA*, *tms2* and *tmr*) located on T-DNA after

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integration in the plant genome transcribe auxin and cytokinin in transformed cells and uncontrolled and unbalanced synthesis of these hormones lead to tumor production (Shaw *et al.*, 1988; Pzour and Das, 1990; Shurvinton and Ream, 1991). *A. tumefaciens* has been widely used for transformation of plant cells (Horsch *et al.*, 1985, 1988; An, 1985; An *et al.*, 1988). The transfer of T-DNA to plant cells has been demonstrated by *A. tumefaciens* co-cultivation with plant protoplast (Depicker *et al.*, 1985), callus suspension (An *et al.*, 1988) and leaf discs (Horsch *et al.*, 1988). Temperature sensitive step is associated with *vir* region induction (Tempe *et al.*, 1977; Alt-Moerbe *et al.*, 1988; Kojima, 1990; Jin *et al.*, 1993). which ultimately control the T-DNA transfer to plant cells. At higher temperature *vir* gene expression is specially inhibited (Kojima, 1990; Jin *et al.*, 1993). The VirA and VirG protein products are involved in the induction of Ti plasmid virulence operon (Shaw *et al.*, 1988; Jin *et al.*, 1990) and VirA protein is the molecule which senses temperature (Chang and Winans, 1992). Various studies have shown that *A. tumefaciens* causes tumors on plants only at temperature below 32°C and virulence gene expression is specifically inhibited at temperature above 32°C (Kojima, 1990; Jin *et al.*, 1993). Our results on transformation efficiency of different indigenous strains of *A. tumefaciens* revealed that co-cultivation time also have impact on transformation efficiency (Qazi, 1996; Qazi and Hasnain, 1996). Hence the effects of co-cultivation time and temperature on the transformation efficiency of seventeen *A. tumefaciens* strains were investigated via callus co-cultivation and explant transformation. Results of these experiments are being reported here.

MATERIALS AND METHODS

Seventeen *Agrobacterium tumefaciens* strains, AM1, CF3, CD1, EI1, EI2, EI3, Pg2, MA5, MA6, Ma4, MI2, MI3, MI4, PR1, ST3, ST4 and TC1 were used in this study. Isolation and characterization of these strains has been described elsewhere (Qazi, 1996; Qazi and Hasnain, 1996). For growth of *A. tumefaciens* strains potato agar (Gerhardt *et al.*, 1994), nutrient dextrose agar and potato dextrose agar (Lelliott and Stead, 1987) media were used. Callus of *Brassica oleracea* L. was obtained from sterilized seeds on MS medium (Murashige and Skoog, 1962) containing 2,4-D (2,4-dichlorophenoxy-acetic acid, 4 mgl^{-1}) and coconut milk (10%). The transformation of callus and callus suspension cultures was accomplished via co-cultivation technique of An *et al.* (1988). The callus and callus suspension cultures were co-cultivated with liquid cultures, adjusted to 4-5x10^8 cells ml^{-1}, of *A. tumefaciens* strains. MS media with and without auxins was used for callus cultures, supplemented with kanamycin (100 μgml^{-1}) and carbenicillin (500 μgml^{-1}) whenever required, in illuminated light (16 hours, 3 K lux) at 25±1°C. Five plants, *Capsicum annuum* L., *Lycopersicon esculentum* Mill., *Medicago falcata* L., *Raphanus sativus* L. and *Spinacea oleracea* L., were used for explant experiments. Seeds of these plants were procured from Punjab Seed Corporation. Plant seeds were sterilized in 10% commercial sodium hypochlorite for 5 minutes. Thoroughly washed disinfected seeds were grown in autoclaved Petri plates lined with double layer of filter paper, containing 5 ml of sterilized glass distilled water in illuminated light (15 hours, 3 K lux) at 25±1°C. For explants (leaf discs, stem cuttings and root cuttings) transformation experiment was carried out following Horsch *et al.* (1988). The explants were co-cultivated with liquid cultures, adjusted to 4-5x10^8 cells ml^{-1}, of *A. tumefaciens* strains. The co-cultivated explants were blot dried and

transferred to auxin free MS medium (supplemented with carbenicillin 300 μgml^{-1} and kanamycin 100 μgml^{-1}) in illuminated light (16 hours, 3 K lux) at 25±1°C. Different combinations of time (1, 3 and 10 minutes) and temperatures (24°, 28° and 37°C) were used for co-cultivation of callus suspension cultures of *B. oleracea*, freshly excised callus pieces of *B. oleracea* and explant (leaf discs, stem cuttings and root cuttings). Auxin independent growth was the criteria for positive transformation response. Observations were made daily and changes were regularly recorded.

RESULTS

Co-cultivation of Brassica oleracea callus suspension

The callus suspension of *B. oleracea* were co-cultivated with the seventeen *A. tumefaciens* strains for three time intervals (1, 3 and 10 minutes time duration) at three different temperatures (24°, 28° and 37°C). The co-cultivated callus suspension cultures (with *A. tumefaciens* strains), along with control (treated with dilution medium for same time and temperature), were initially transferred to MS medium containing 2,4-D (4 mgl^{-1}) and coconut milk (10%) supplemented with antibiotics, carbenicillin 500 $\mu g\ ml^{-1}$ and kanamycin 100 $\mu g\ ml^{-1}$. At the end of second week all the co-cultivated calluses were transferred to auxin free MS medium without antibiotics. The calluses co-cultivated for 1 minute time duration at 24°C showed no transformation response on auxin free MS medium and by the end of fourth week in these calluses signs of browning was exhibited (Table I). Similar response was observed in the callus suspension cultures co-cultivated for 1 minute time duration at 37°C *i.e.*, lack of transformation response, but these calli showed symptoms of browning by the end of second week. In some of these calli signs of necrosis was also observed during the fourth week of incubation on auxin free MS medium (Table I). The calli co-cultivated for 1 minute time duration at 28°C showed transformation and proliferation response on auxin free MS medium (Table I). Different responses were shown by the callus suspension co-cultivated with different strains. The callus suspensions co-cultivated with AM1, EI1, EI2, EI3, MI2, MI3 and MI4 gave early callus proliferation response as compared to the rest of the strains. These strains gave transformation response during the second week of incubation on auxin free MS medium. The CD1 and MA5 co-cultivated callus suspensions showed slow callus proliferation during the second week on auxin free MS medium but during the third week of transfer on auxin free medium a rapid callus proliferation was observed in both cases. The CF3, Pg2, MA6, Ma4, PR1, ST3, ST4 and TC1 showed delayed transformation response and slow callus proliferation. These strains gave callus proliferation response during the third week of transfer on auxin free MS medium, except MA6 and TC1 which showed callus proliferation response during the fourth week of incubation. The callus suspensions co-cultivated for 1 minute time duration at 28°C with AM1, EI2 and EI3 also exhibited rhizogenesis response during the third week of incubation on auxin free MS medium (Fig.1a). In AM1 co-cultivated calluses rhizogenesis was followed by caulogenesis (Fig.1c).

The callus suspensions co-cultivated for 3 minutes duration at 24°C with AM1, CD1, EI1, EI2, EI3, MA5, MI2, MI3 and MI4 showed transformation response but this transformation response was delayed and callus proliferation was rather slow as

compared to the transformation response of callus suspensions co-cultivated for 1 minute duration at 28°C and callus suspensions co-cultivated for 3 minutes duration at 28°C (Table I). The callus suspensions co-cultivated for 3 minutes duration at 24°C (with AM1, CD1, EI1, EI2, EI3, MA5, MI2, MI3 and MI4) gave transformation response during the third week of incubation on auxin free MS medium. The rest of the strains co-cultivated for 3 minutes time duration at 24°C showed no transformation response on auxin free MS medium (Table I). The callus suspensions co-cultivated with AM1, EI2 and EI3 (for 3 minutes at 24°C) also exhibited rhizogenesis during the fifth week of incubation (Fig.1b). The unbranched and hair like rhizoids were produced on the general surface of calli in a localized regions (Fig.1b). Whereas in the callus suspensions co-cultivated for 1 minute duration at 28°C rhizoids were formed on small protuberance like structures and the tip of protuberance was without any rhizoids (Fig.1a). The rhizoids produced on the protuberance were many fold greater in number (Fig.1a) than the rhizoids formed in the localized region of calli co-cultivated for 3 minutes duration at 24°C (Fig.1b). The callus suspensions co-cultivated for 3 minutes duration at 28°C showed transformation response in all the co-cultivated cases (Table I). However, different strains showed different transformation responses. The callus suspensions co-cultivated with AM1, CD1, EI1, EI2, EI3, MA5, MI2, MI3 and MI4 showed callus proliferation response during the second week of incubation on auxin free MS medium. The CD1 and MA5 co-cultivated callus suspensions showed slow callus proliferation response during the second week but in the third week the calli proliferated quickly. The rest of the strains co-cultivated for 3 minutes duration at 28°C showed transformation response during the third week of incubation, except MA6 and TC1. The MA6 and TC1 showed callus proliferation during the fourth week of incubation on auxin free MS medium. The callus suspensions co-cultivated for 3 minutes duration at 28°C showed better callus proliferation response as compared to the callus suspensions co-cultivated for 1 minute duration at 28°C. The callus suspensions co-cultivated with AM1, EI2 and EI3 (for 3 minutes at 28°C) showed rhizogenesis response similar to that shown by the callus suspensions co-cultivated for 1 minute time duration at 28°C (Fig.1a). In AM1 co-cultivated calli (for 3 minutes at 28°C) caulogenesis was also observed during the fifth week of incubation on auxin free MS medium (Fig.1c).

The callus suspensions co-cultivated for 10 minutes time duration at 24° and 37°C showed almost similar responses. In all cases no callus proliferation response on auxin free MS medium was observed (Table I) and the co-cultivated calluses become brown and necrotic during the sixth week of incubation. The callus suspensions co-cultivated with AM1, CD1, EI1, EI2, EI3, MA5, MI2, MI3 and MI4 for 10 minutes time duration at 28°C showed some callus proliferation response during the second week and third week of incubation on auxin free MS medium. But afterward growth in these calli was arrested followed by the browning of cells and ultimately necrosis was observed during the sixth week of incubation. The rest of the strains showed no transformation response and cells of callus suspensions co-cultivated with these strains ultimately become brown and necrotic. All the control callus suspensions showed no callus proliferation in any case (Table I). The control cultures showed signs of browning during the third week of incubation on auxin free MS medium. This browning response continued and ultimately necrosis started in the fourth week of incubation (Fig.1d).

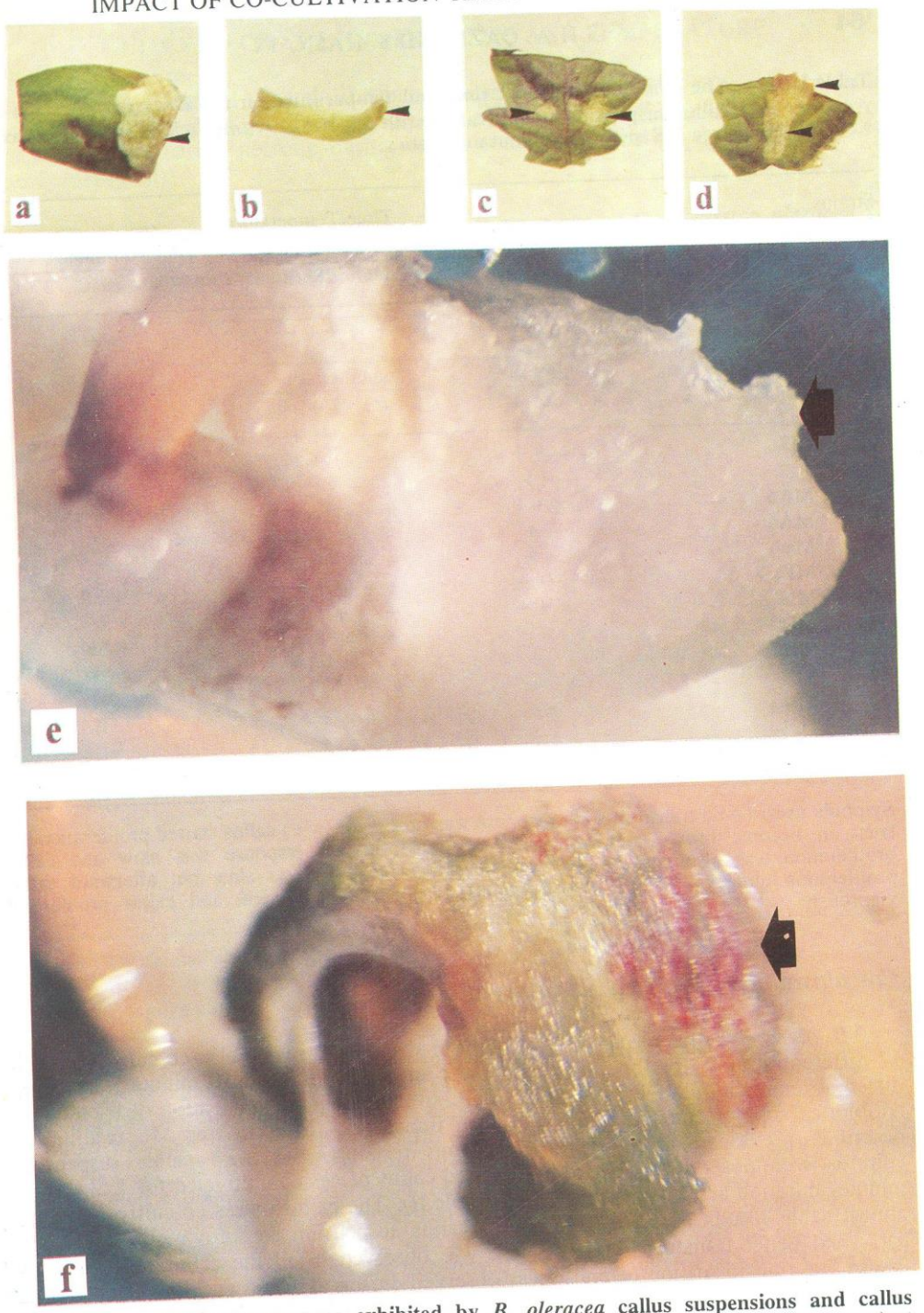

Fig. 1: Various responses exhibited by *B. oleracea* callus suspensions and callus pieces co-cultivated for three different time intervals (1, 3 and 10 minutes) at three different temperatures (24°, 28° and 37°C). a) Rhizogenesis on protuberances in a localized region; b) rhizogenesis on the general surface in a localized region; c) caulogenesis; d) control showing browning and necrogenesis.

Table I: **The combined effect of time and temperature on the *Brassica oleracea* L. callus suspension co-cultivation with selected *A. tumefaciens* strains on auxin free MS medium without antibiotics.**

Strains		Time/Temperature								
		1 Minute			3 Minutes			10 minutes		
		24°C	28°C	37°C	24°C	28°C	37°C	24°C	28°C	37°C
1.	AM1	-	++	-	+	+++	-	-	w+	-
2.	CF3	-	+	-	-	+	-	-	-	-
3.	CD1	-	++	-	+	++	-	-	w+	-
4.	EI1	-	++	-	+	+++	-	-	w+	-
5.	EI2	-	++	-	+	+++	-	-	w+	-
6.	EI3	-	++	-	+	+++	-	-	w+	-
7.	Pg2	-	+	-	-	+	-	-	-	-
8.	MA5	-	++	-	+	++	-	-	w+	-
9.	MA6	-	+	-	-	+	-	-	-	-
10.	Ma4	-	+	-	-	+	-	-	-	-
11.	MI2	-	++	-	+	+++	-	-	w+	-
12.	MI3	-	++	-	+	+++	-	-	w+	-
13.	MI4	-	++	-	+	+++	-	-	w+	-
14.	PR1	-	+	-	-	+	-	-	-	-
15.	ST3	-	+	-	-	+	-	-	-	-
16.	ST4	-	+	-	-	+	-	-	-	-
17.	TC1	-	+	-	-	+	-	-	-	-
18.	Control	-	-	-	-	-	-	-	-	-

Symbols uses: (-) no transformation response was observed; (w+) callus started proliferation but later on become brown and necrotic; (+) transformation response was slow and callus proliferation was also slow; (++) transformation response was slow but afterward callus proliferation quickly; (+++) transformation response was efficient and callus proliferated vigorously.

Co-cultivation of Brassica oleracea callus

B. oleracea callus pieces of 2 to 3 mm in size (~1 mg) were also co-cultivated with 17 different strains, for three different times intervals (1, 3 and 10 minutes) and three different temperatures (24°, 28° and 37°C), followed by the eradication of bacteria on antibiotics supplemented medium and then transferred to auxin free MS medium as described previously. Similar transformation responses, as with callus suspension cultures, were observed in the freshly excised callus pieces co-cultivated for 1, 3 and 10 minutes duration at 24°, 28° and 37°C (Table II). The callus pieces co-cultivation gave almost same results as were observed in callus suspension co-cultivations. However, early and better transformation response was observed in all the callus co-cultivation cases. The transformation response was observed during the first week of incubation on auxin free MS medium in contrast to callus suspension cultures. However, no transformation response was observed in calluses co-cultivated for 1 and 10 minutes

duration at 24°C and 1, 3 and 10 minutes time duration at 37°C (Table II). All the control calli showed no proliferation response on auxin free MS medium. The control calli started appearing necrotic during fourth week of incubation on auxin free MS medium (Fig. 1d).

Table II: **The combined effect of time and temperature on the *Brassica oleracea* L. callus co-cultivation with selected *A. tumefaciens* strains on auxin free MS medium without antibiotics.**

Strains		Time/Temperature								
		1 Minute			3 Minutes			10 minutes		
		24°C	28°C	37°C	24°C	28°C	37°C	24°C	28°C	37°C
1.	AM1	-	++	-	+	+++	-	-	w+	-
2.	CF3	-	+	-	-	+	-	-	-	-
3.	CD1	-	++	-	+	++	-	-	w+	-
4.	EI1	-	++	-	+	+++	-	-	w+	-
5.	EI2	-	++	-	+	+++	-	-	w+	-
6.	EI3	-	++	-	+	+++	-	-	w+	-
7.	Pg2	-	+	-	-	+	-	-	-	-
8.	MA5	-	++	-	+	++	-	-	w+	-
9.	MA6	-	+	-	-	+	-	-	-	-
10.	Ma4	-	+	-	-	+	-	-	-	-
11.	MI2	-	++	-	+	+++	-	-	w+	-
12.	MI3	-	++	-	+	+++	-	-	w+	-
13.	MI4	-	++	-	+	+++	-	-	w+	-
14.	PR1	-	+	-	-	+	-	-	-	-
15.	ST3	-	+	-	-	+	-	-	-	-
16.	ST4	-	+	-	-	+	-	-	-	-
17.	TC1	-	+	-	-	+	-	-	-	-
18.	Control	-	-	-	-	-	-	-	-	-

For details of symbols used, see Table I.

Explant co-cultivation

The effects of co-cultivation time and temperature on the transformation efficiency of seven *A. tumefaciens* strains (which gave better transformation responses in callus/callus suspension co-cultivation experiments) in the explants (leaf discs, stem cuttings and root cuttings) of five different plant species was studied for three time intervals (1, 3 and 10 minutes) at three different temperatures (24°, 28° and 37°C). The co-cultivated explants, along with control (treated with dilution medium for same time interval and temperature), were transferred to auxin free MS medium supplemented with antibiotics, carbenicillin 300 μg ml^{-1} and kanamycin 100 μg ml^{-1}. At the end of second week the co-cultivated explants were transferred to fresh auxin free MS medium without antibiotics. The explants (leaf discs, stem cuttings and root cuttings) co-cultivated for 1, 3 and 10 minutes time interval at 24°, 28° and 37°C. All the explants of five different

plant species used for co-cultivation with *A. tumefaciens* strains for 1 and 10 minutes time duration at 24°, 28° and 37°C showed no transformation response on auxin free MS medium without antibiotics. The explants (stem cuttings and root cuttings) co-cultivated for 3 minutes time duration at 24°, 28° and 37°C also exhibited no transformation response on auxin free MS medium except for one case i.e., with AM1 co-cultivation, in which stem cutting of *Capsicum annuum* showed some callus like cells proliferation at the excised edge. The leaf discs co-cultivated for 3 minutes time interval at 37°C showed no transformation response while leaf discs co-cultivated for 3 minutes duration at 24°C gave positive transformation response only in case of *Lycopersicon esculentum* leaf discs co-cultivated with AM1, EI2 and EI3. With rest of the strains no transformation response was exhibited even in *L. esculentum*.

Out of these five plants used as explant in the co-cultivation experiments T-DNA transfer and expression was manifested only in four plants. In *C. annuum* only leaf discs co-cultivated at 28°C for 3 minutes time interval showed transformation response. The leaf discs co-cultivated with AM1, EI2 and EI3 showed transformation response during the second week of transfer on auxin free MS medium without antibiotics. The leaf discs formed callus like mass of cells at one of its excised end (Fig.2a). The stem cutting of *C. annuum* also showed some transformation response, when co-cultivated with AM1 at 28°C for 3 minutes time duration where some cells at the excised end produced small callus like mass of cells (Fig.2b). This was the only case in which stem cutting showed transformation response. All other strains were unable to show any transformation response in stem cuttings of *C. annuum*.

In *L. esculentum* only leaf discs co-cultivated for 3 minutes time duration at 24° and 28°C showed transformation response. The leaf discs co-cultivated (for 3 minutes at 24°C) with AM1, EI2 and EI3 showed transformation response during the second week of incubation on auxin free MS medium. The leaf discs formed small callus like mass of cells at localized regions (Fig.2c). The leaf discs co-cultivated with AM1, EI2, EI3, MI2, MI3 and MI4 for 3 minutes time duration at 28°C also produced callus like mass of cells at the excised edges. The leaf discs co-cultivated at 28°C produced better callus proliferation response as compared to the leaf discs co-cultivated at 24°C (Fig.1d). No transformation response was observed in any other co-cultivated explant. No transformation response was observed in co-cultivated explant of *M. falcata* with any strain. The co-cultivated explants lost their green coloration and started decaying on the auxin free MS medium in the fourth week of transfer to auxin free MS medium.

In *R. sativus* AM1 and EI3 and in *Spinacea oleracea* AM1 and EI2 co-cultivated leaf discs, at 28°C for three minutes time duration, showed transformation response on auxin free MS medium. The *R. sativus* leaf discs lost their green coloration at the end of first week of transfer on auxin free MS medium and during the second week produced callus like mass of cells from the excised edge. The callus like mass of cells were compact and white in color (Fig.2e). All other strains showed no transformation response in *R. sativus* explants. While the *S. oleracea* leaf discs did not lost their coloration on auxin free MS medium and also produced callus like mass of cells. These cells were soft and greenish in color (Fig.2f). The rest of the strains showed no positive transformation response in leaf discs as well as in all other explants of the *S. oleracea*.

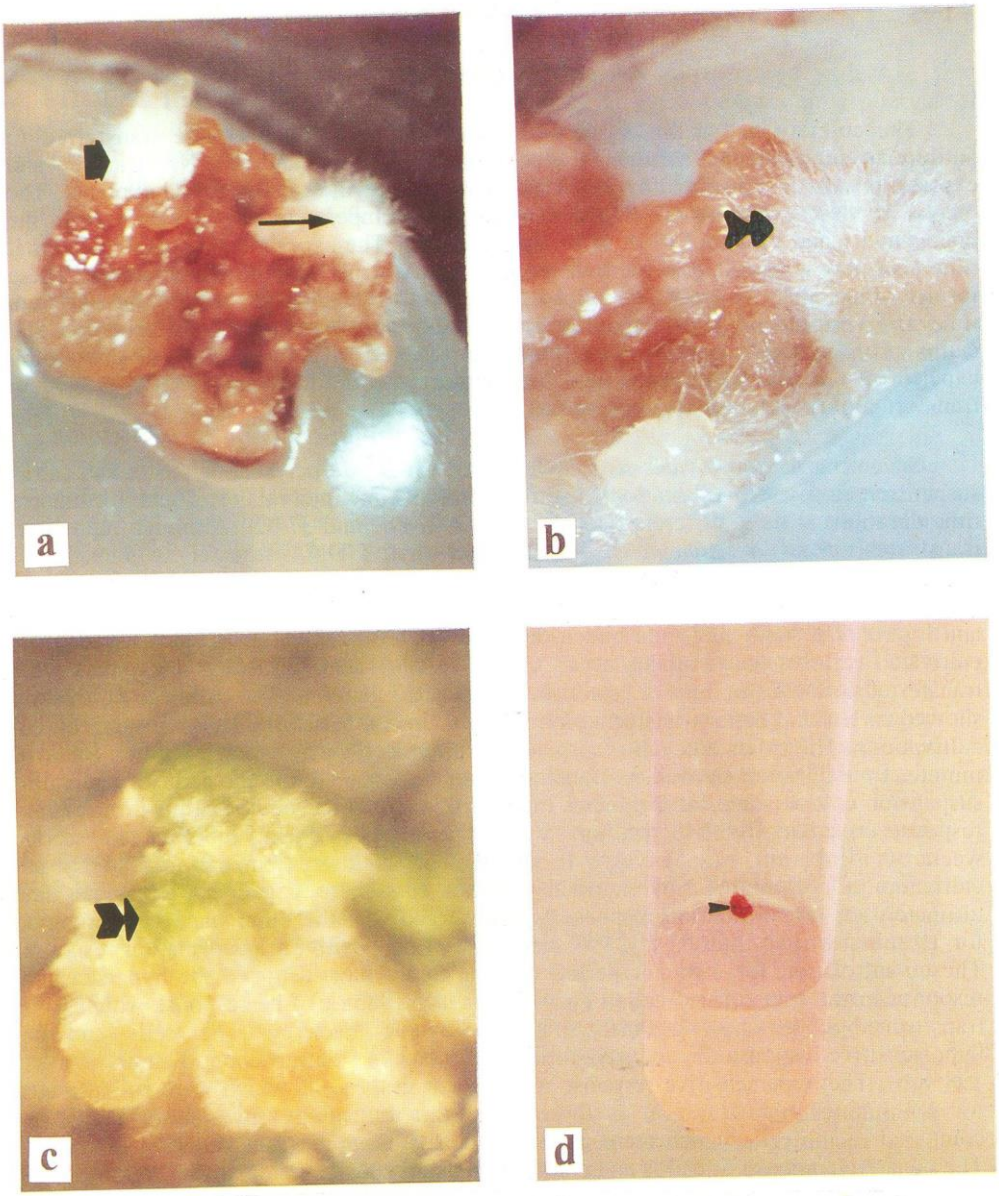

Fig. 2: Various responses exhibited by explants co-cultivated for three different time intervals (1, 3 and 10 minutes) at three different temperatures (24°, 28° and 37°C). a) Callus like mass of cells produced at the excised edge of leaf disc of *C. annuum*; b) some cells exhibiting proliferation response at the edge of stem explant of *C. annuum*; c) small tumors induced on leaf disc of *L. esculentum* co-cultivated at 24°C for 3 minutes time interval; d) somewhat larger tumors induced on leaf disc of *L. esculentum* co-cultivated at 28°C for 3 minutes time interval; e) white compact callus like mass of cells induced on *R. sativus* leaf disc; f) soft greenish callus like cells formed on *S. oleracea* leaf disc.

DISCUSSION

The co-cultivated callus suspensions, calluses and explants were transferred to antibiotics supplemented media to eradicate the bacteria and T-DNA transfer and expression was demonstrated by auxin independent growth of calluses and explants. The results of these experiments showed that time and temperature play a vital role in the oncogenicity and virulence of *A. tumefaciens* strains. The experimental data showed that time also play a crucial role for transfer of T-DNA at permissible temperatures. These results reflect that for callus co-cultivation as well as explant co-cultivation optimum time and temperature was 3 minutes and 28°C where maximum transformation and callus proliferation responses were observed (Table I, II). In explant co-cultivation experiments at the same time and temperature (3 minutes and 28°C) maximum transformation results were also achieved.

Seventeen *A. tumefaciens* strains were used for *B. oleracea* callus pieces and callus suspension cultures co-cultivation for three different time intervals (1, 3 and 10 minutes time duration) at three different temperatures (24°, 28° and 37°C). Although reports on transformation of *A. tumefaciens* demonstrate that T-DNA transfer is temperature sensitive phenomenon, and temperature above 32°C hinder *vir* gene induction, a prerequisite for transfer of T-DNA. According to Jin *et al.* (1993) at temperature 32°C and higher, the VirA molecule undergoes a reversible inactivation. Hence we also co-cultivated calluses and explants at 37°C and co-cultivated calluses and explants were transferred to 24°C for further establishment in plant genome. The callus co-cultivation showed earlier and better transformation response in comparison to callus suspension co-cultivations. The callus and callus suspension cultures co-cultivated at 24°C for 1 and 10 minutes time duration showed no transformation response. While the calluses and callus suspension co-cultivated for 3 minutes time duration at 24°C showed callus proliferation response on auxin free MS medium (without antibiotics) during the first and second week of incubation, respectively. It shows that at lower temperature (24°C) co-cultivation for 1 minute time interval was not enough to trigger the *vir* genes and ultimately no transformation response was observed. On the other hand co-cultivation for 10 minutes time interval at 24°C also showed no positive transformation response. The co-cultivation for a longer period of time might have actuated the hypersensitive response in the transformed cells (Yanofsky *et al.*, 1985a,b), the response which some transformed cells show against some pathogenic bacteria. The cells showing hypersensitive response die and save the rest of the cells from invasion (Yanofsky *et al.*, 1985a,b). The hypersensitive response was evident in the cells which were co-cultivated for 10 minutes time duration as these cells started turning brown more rapidly as compared to the cells which were co-cultivated for 1 minute time duration (at 24°C). The negative response of callus and callus suspension co-cultivations for 10 minutes time duration (at 24°C) might also be due to co-suppression factor. During co-cultivation several T-DNA copies are transferred to some cells and in some cases these T-DNA copies lead to gene inactivation (Hooykaas and Schilperoort, 1992; Matzke *et al.*, 1989). There might be a possibility that at 24°C the co-cultivation for 10 minutes time interval lead to insertion of several T-DNA copies in the transformed calluses, which ultimately inactivated the gene expression. Alternatively both phenomenon, hypersensitive response and co-suppression effect, might be responsible for the negative transformation response of the co-cultivated callus pieces and callus suspensions on

auxin free MS medium. The callus and callus suspensions co-cultivated with AM1, CD1, EI1, EI2, EI3, MA5, MI2, MI3 and MI4 at 24°C for 3 minutes time interval showed callus proliferation on auxin free MS medium during the second and third week of incubation, respectively. Here response was delayed and calluses proliferation was slow as well. In callus and callus suspension co-cultivations at 24°C for 3 minutes time rhizogenesis was also delayed and rhizoids were produced on general surface of the proliferating callus (Fig.1b).

The callus suspensions and calluses co-cultivated at 28°C for 1 and 3 minutes time duration gave better transformation response as compared with callus transformation response observed at 24°C (for 3 minutes time interval). This response shows that 1 and 3 minutes co-cultivation time at 28°C were suitable for *vir* region induction and T-DNA transfer in the co-cultivated cells. The rhizogenic response was more vigorous at this temperature and with AM1 co-cultivation at this temperature pronounced caulogenic response was also observed. It seems that this time and temperature are not only optimum for *vir* gene induction, but they might also influence subsequent transformation mechanism/s. At this time and temperature the co-cultivation of callus and callus suspension might activate insertion elements present on T-DNA. The insertion elements play a role in the abnormal production of auxin and cytokinin (Ponsonnet *et al.*, 1995). On the other hand the flanking DNA and chromatin structure also effect the expression of T-DNA genes in different transformed cells (Horsch *et al.*, 1988). Certain mutations in the transferred T-DNA also effect the tumor morphology of the transformed cells (Gheysen *et al.*, 1985). Mutations in the 'shooty' locus (*shi* or *tms*) produced abnormal shoot formation in the transformed cells. Whereas mutations in the 'rooty' locus (*roi* or *tmr*) caused abnormal development of roots instead of production of undifferentiated mass of cells. Unusual larger tumor formation as compared to normal tumor induction is caused by the mutation in the morphogenetic locus, *tml* (Grierson and Covey, 1988). However at 28°C a longer period of co-cultivation (10 minutes) initially showed some proliferation response on auxin free MS medium (Table I, II) but the proliferation response was accompanied by the browning of the cells, which dominated the callus proliferation. These observations demonstrate that co-cultivation for a longer period of time stir the hypersensitive response (Yanofsky *et al.*, 1985a,b) in the transformed cells (Table I, II). CF3, MA6, Ma4, PR1, ST3, ST4 and TC1 gave no transformation response when co-cultivated for 10 minutes time interval at 28°C. This might be due to more pronounced hypersensitive response of the co-cultivated cells (Yanofsky *et al.*, 1985a,b) and cells could not be survived after co-cultivation.

The callus suspensions and calluses co-cultivated for 1, 3 and 10 minutes duration at 37°C gave no transformation response. At higher temperature virulence gene expression is specially inhibited (Jin *et al.*, 1993; Kojima, 1990). Chang and Winans (1992) reported that the molecule which senses temperature is VirA protein. *In vitro* phosphorylation experiments clearly demonstrated that the function of VirA protein, autophosphorylation and phosphate transfer to VirG, are inhibited at higher temperatures (Jin *et al.*, 1993). Hence at 37°C temperature inhibition in *A. tumefaciens vir* gene induction resulted in negative transformation response of the co-cultivated calluses. These results reflect that even co-cultivation for 1 minute at 37°C inhibit T-DNA transfer or subsequent steps in transformation. Although treated calluses were shifted to 24°C but damage at 37°C was irreversible. These results are contradictory to the report

of Jin *et al*. (1993).

In explant co-cultivation experiments similar responses, as in callus/callus suspensions, were observed. The higher temperature gave no transformation response due to inhibition of *vir* genes activities. However, lower temperature (24°C) also gave, almost, no transformation response except with AM1, EI2 and EI3 strains where some transformation response was observed. These strains gave transformation response in *L. esculentum* leaf discs co-cultivated for 3 minutes time interval at 24°C. These results suggest that either plant become resistant to infection by *A. tumefaciens* at lower temperature (24°C) or some other factors might be involved in the negative transformation response. The explants, stem cuttings (except one case) and root cuttings showed no transformation response. The only transformation response was observed in the stem cutting of *C. annuum* when co-cultivated with AM1 where the response was also not very pronounced (Fig.1b). The cells of these explants (stem and root cuttings) might be more resistant to *A. tumefaciens* strains. The leaf discs co-cultivated at 28°C for 3 minutes exhibited better transformation response as in case of callus/callus suspension co-cultivations.

These results clearly demonstrated that virulence of *A. tumefaciens* is also dependent on time and temperature. At higher temperature *A. tumefaciens* virulence gene expression is inhibited and ultimate result is negative transformation response. Jin *et al*. (1993) suggested that at higher temperature the plant become more resistant to infection by *A. tumefaciens* and/or functions of some *vir* gene products are lost. It is not only the inability of the *A. tumefaciens* but other plant factors also play role in the negative transformation response (Jin *et al*., 1993). Another possibility is that the plant factor required for *A. tumefaciens* transformation may be temperature sensitive. The higher (37°C) and lower (24°C) temperatures might shut down the synthesis of proteins required for *A. tumefaciens* attachment or transformation. Co-cultivation even for 1 minute at higher temperature caused irreversible damage. The three strains AM1, EI2 and EI3 showed efficient, better and maximum transformation responses in all the different time intervals and temperatures (except 37°C) used for co-cultivation of callus and callus suspensions. These strains also showed transformation response in explant (leaf discs) at 28°C as well as 24°C exhibiting wider temperature tolerance range for *vir* gene induction. These results reflect that these strains efficiently transfer the T-DNA at both the temperatures. The VirA protein of these strains might work equally well at 28°C and 24°C as compared to the rest of strains. A thorough knowledge of Ti plasmids, present in these strains, is required to know the actual mechanism involved.

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