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Response of stem explants to screening and explant source as a basis for methodical advancing of regeneration protocols for chrysanthemum

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Abstract

Logical advancing of regeneration protocols as an end result of response to screening and use of alternate explants is reported. Stem explants screened on medium 1 (MS with 1 mg l^{-1} BAP and 0.1 mg l^{-1} IAA) produced 4 responses and were classified as Group 1: more than 1.6 shoots per explant; Group 2: less than 1.6 shoots per explant; Group 3: only callus and Group 4: no response. To regenerate groups 2, 3 and 4, specific media were developed (media 2 a,b, media 3 a,b and media 4 a–i). Media 2 and 4 had changes in hormonal combination compared to medium 1, while medium 3 had additional changes in vitamins. Nineteen out of the 37 cvs. tested were regenerated on stem explants, based on the response to screening technique. Cultivars recalcitrant on stem explants even after exposure to modified media were reinvestigated on leaf. High concentration hormone pulse as induction (medium 5) followed by a modified regeneration medium (medium 6d) ended in successful regeneration of leaf explants. Four out of the 14 cvs. recalcitrant on stem were successfully regenerated using leaf explants. Response-based advancing of regeneration protocols in addition to use of alternate explants for recalcitrant cultivars resulted in efficient regeneration of 23 of the 37 cvs. on assessment.

Abbreviations: BAP – benzylaminopurine; cvs. – cultivars; GA3 – gibberelic acid; IAA – indole acetic acid; MES – 2 (N-morpholino) ethanesulfonic acid; NAA – naphthylene acetic acid

Introduction

Regeneration has been reported for several cvs. of chrysanthemum of the species *Dendranthema grandiflora* (Anderson, 1987). Successful regeneration has been reported using different explants like flower receptacle (Hill, 1968), shoot tips (Earle and Langhans, 1974), pedicel (Roest and Bokelmann, 1975), petal segments (Bush et al., 1976) and leaf (Slusarkiewicz et al., 1981; Bhattacharya et al., 1990; Kaul et al., 1990).

A drawback of most protocols is their cultivar specificity, as an effect of which regeneration of a new cultivar is by trial and error. The problem is further complicated as most chrysanthemums are hexaploids and are developed via mutation breeding techniques. Regeneration depends on expression of endogenous hormone genes which could be affected by these mutations, like in cotton (Chen et al., 1996). Mutations can bring about change in hormone concentrations and/or changes in sensitivity to hormones (Reid and Davies, 1987). Strong intercation between nucleus and cytoplasm in influencing regeneration in sunflower has been observed (Nestares et al., 1998). Thus regeneration is a result of the combined effect of endogenous hormone gene expression and responsiveness to external stimuli.

Direct organogenesis protocol used for *Agrobacterial* transformation (De Jong et al., 1994) was used as medium for screening stem explants. Subsequently altering the inorganic and organic constituents paved

the way for development of new protocols. To regenerate cvs. recalcitrant on stem, we tried leaf explants, successfully used in green bean (Franklin et al., 1993), tomato (Van Roekel et al., 1993), apple (De Bondt, 1996), begonia (Kiyokawa et al., 1996), kiwi (Yamakawa and Chen, 1996) and chrysanthemum (Slusarkiewicz et al., 1981; Bhattacharya et al., 1990; Kaul et al., 1990). The principle behind changing the explant is to make use of the differences in levels of endogenous hormones which effect regeneration like in poplar (Sasamoto et al., 1995).

We focussed our aims in the current investigation on (1) providing a methodical tissue culture technique for a large number of cvs., (2) the use of response to screening as a basis in addition to (3) the use of alternate explants (leaf) for cvs. recalcitrant on stem.

Materials and methods

Experimental layout

Every experiment had four replications in four petridishes with ten explants per dish. The data analysed is presented in tables as number of shoots per explant. Cultivars producing 1.6 shoots per explant were considered good based on our experience and requirement for efficient transformation.

Plant material and surface sterilisation

Experiments were based on 37 cvs. obtained from breeders, listed in Table 2. Material was grown in the greenhouse under standard conditions (Machin and Scopes, 1978). Stem explants were obtained from the first two internodes of three-week-old shoot tips while leaf explants from the third and fourth position. The shoots tips, and leaves, were surface sterilised in 1% (W/V) hypochlorite for 20 min and rinsed in sterilised water thrice for 15 min.

Stem explants

Subsequent to the surface sterilisation, the shoot tips were sliced across their length to obtain slices of 2–4 mm. Slices were placed distal side down on medium 1 and transferred to climate rooms maintained at 25 °C with a 16-h photoperiod. The dishes were covered with a white cloth to keep the light level at 4 μmolm^{-2} s^{-1}.

Leaf explants

The leaves were cut 0.5 cm away from the midrib on either side, followed by a slicing of the midrib resulting in two strips. The strips were cut across their length to produce explants of 0.5 cm^2. This ensured a high degree of uniformity of the explant with four cut-edges, with a sliced midrib on one side. Explants were placed abaxial side down on medium 5 and transferred to the climate room.

Media

The screening of stem explants was on medium 1 (de Jong et al., 1994, 1995) composed of: MS (Murashige and Skoog, 1962) salts + vitamins, 1 mg l^{-1} BAP, 0.1 mg l^{-1} IAA, 30 g l^{-1} sucrose and 7 g l^{-1} plant tissue culture agar. The screening of leaf explants was on medium 5 (Gutterson et al. 1994, MS salts, B5 vitamins, 2 mg l^{-1} BAP, 1 mg l^{-1} NAA, 7 g l^{-1} tissue culture agar, 30 g l^{-1} sucrose and 3 mm MES). Based on the response, medium 5 was limited to induce the leaf explants. Further regeneration after induction was on medium 6d (Table 6). A summary of the media used are presented in Table 1 and the movement of cultivars from one medium to another are described in Figure 1.

Regeneration and phenotype of cultured explants

Stem explants
Shoot primordia were counted, 13 days after the initiation of culture, and recorded as number of shoot primordia per explant. After 21 days, the explants were transferred to fresh medium. At the end of 42 days the microshoots were harvested and recorded as number of harvestable shoots per explant. Based on the response of the stem explants to medium 1 (number of harvestable shoots, callus formation or none) cvs. were classified into four groups (Table 2).

Modification of regeneration protocols for stem explants

Group 2
To regenerate the cvs. that produced less than 1.6 shoots per explant (Group 2), only the BAP concentrations in the medium 1 (1 mg l^{-1}) were either lowered to 0.1 mg l^{-1} or increased to 2 mg l^{-1} (media 2 a,b). Recalcitrant cultivars that failed on the media 2 a,b were retested using leaf explants.

Table 1. Different media tested for different groups of stem and leaf explants of chrysanthemum

Media	Media number	Vitamins	Auxin mg l^{-1}	Cytokinin and other components mgl^{-1}
Group 1	1	MS	IAA 0.1	BAP 1
Group 2	2a	MS	IAA 0.1	BAP 0.1
Group 2	2b	MS	IAA 0.1	BAP 2.0
Group 3	3a	B5	IAA 0.1	Kinetin 1 Coconut water 10W/V
Group 3	3b	B5	IAA 0.1	Kinetin 1 GA 3 10
Group 4	4a	MS	NAA 1	BAP 1
Ind. media (leaf)	5	B5	NAA 1	BAP 2 3 mM MES buffer
Reg. med (leaf)	6d	B5	–	BAP 0.25 3 mM MES buffer

All media contain MS salts, 30 g l^{-1} sucrose and 7g l^{-1} plant tissue culture agar. Hormones mentioned are mg per litre. These were the modifications developed for each group with the aim of regenerating them.
Key – Ind. media: Induction media for four days. Reg. med: Regeneration media.

Table 2. Characterisation and classification of 37 cultivars based on the response to regeneration in initial screening on media number 1

Cultivar	Primor	Shoot	Callus	Concl	Cultivar	Primor	Shoot	Concl	Callus
Cleo	50	4.6	–	Group 1	Roubiax	4	2	Group 2	–
Rush	40	3.6	–	Group 1	Sheba	2	1	Group 2	–
Kris	22	2.8	–	Group 1	Alencon	2	0.7	Group 2	–
CBA 1403	28	2.6	–	Group 1	Granada	2	0.7	Group 2	–
Albert Hein	24	2.3	–	Group 1	Napoli	2	0.5	Group 2	–
Venezia	23	2.1	–	Group 1	Le mans	30	0	Group 2	–
Toulouse	23	2.1	–	Group 1	Mike	0	0	Group 3	+++
Tr. 1	22	2.0	–	Group 1	Hugo	0	0	Group 3	+++
Mayfair	20	1.8	–	Group 1	Money maker	4	0	Group 3	+++
Lineker	20	1.6	–	Group 1	Tr. 2	0	0	Group 3	+++
Tornato	18	1.6	–	Group 1	Tr. 5	0	0	Group 3	+++
Biarritz	20	1.6	–	Group 1	Rubakest	0	0	Group 3	+++
Cocarde	10	8	–	Group 2	Petra	0	0	Group 3	++
Tigerrag	7	5	–	Group 2	Funshine	0	0	Group 3	+
David	6	4.5	–	Group 2	Poso doble	0	0	Group 3	+
Stallion	6	4	–	Group 2	CBA 2053	0	0	Group 4	Nr
XW 109	4	4	–	Group 2	Rainbow	0	0	Group 4	Nr
Tr. 3	4	3	–	Group 2	Tr. 4	0	0	Group 4	Nr
Tourmalin	3	2	–	Group 2					

The numbers of harvestable shoots are per explant obtained as an average value of four replications, with each replication consisting of 10 explants. Based on the phenotype of the explant the cultivars were easily grouped for further investigation.
Key – Primor: The number of primordia per petridish. Shoot: The number of harvestable shoots per petridish. Callus: (– completely absent, + little, ++ moderate, +++ excessive). Nr: No response. (cvs. not producing shoots, callus or primordia).
Group 1: More than 16 harvestable shoots (2 cm) per petridish. Group 2: Less than 16 harvestable shoots per petridish. Group 3: Producing only callus. Group 4: Has no response.

Table 3. The response of 13 cultivars of Group 2 tested on two different concentrations of BAP (0.1 and 2.0 mg l^{-1}), with 1.0 mg l^{-1} as control

Cultivar	Shoot 0.1 BAP Media 2a	Shoot 1.0 BAP Media 1	Shoot 2.0 BAP Media 2b	0.1 vs. 1.0	0.1 vs. 2.0	1.0 vs. 2.0
David	0	0.45	0	***		***
Stallion	0.5	0.4	0.4	*	**	***
Xw 109	1.6	0.4	0.2	***	***	**
Tr. 2	0	0	0.25		**	**
Tr. 3	0	0.3	0	***		***
Lemans	0	0	0.3		*	*
Sheba	1.6	0.1	0	***	***	*
Granada	0	0.075	0.15	***	*	*
Roubijax	0	0.2	0.15	**	*	
Tigerrag	2.5	0.5	0.11	***	***	
Cocarde	0	0.8	0.4	***		**
Tourmalin	0	0.2	0.9	*	**	

The numbers of harvestable shoots are per explant obtained as an average value of four replications, with each replication consisting of 10 explants. A statistical analysis indicating the probability for the response within each cultivar for the media 1, 2a and 2b is also presented.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Group 3

The cvs. that produced only callus (Group 3), were callused on medium 1 for three weeks and transferred to medium 3a (Lemieux et al., 1991, MS salts, B5 vitamins, 1 mg l^{-1} kinetin and 10% W/V coconut water). At the end of three weeks on medium 3a explants were transferred to medium 3b (composed exactly like 3a but coconut water was replaced by 10 mg l^{-1} GA). Cultivars recalcitrant to media 3 a,b were retested using leaf explants.

Group 4

The cvs. which had no response in the screening (Group 4), were tested on media 4 a–i (MS organic and inorganic, with NAA and BAP in nine different combinations, Table 4). Cultivars recalcitrant on media 4 a–i were retested using leaf explants.

Developing a regeneration protocol using leaf explants

Leaf explants of cv. 1581 were induced on medium 5 (Gutterson et al., 1994), for 4, 8 or 16 days, and a statistical programme was run to study its influence (Table 6). Following induction, explants were transferred to nine different regeneration media (MS inorganic, B5 organic with IAA and BAP in 9 different combinations, 6 a–i). The best combination of induction and regeneration (Table 6) was used to test 14 of the 18 cvs. totally recalcitrant on stem explants (Table 7).

The screening of cultivars on stem explants and based on their response their grouping and further retesting on different media and different explants are summarised in a flow chart (Figure 1).

Results

Grouping based on response of stem explants to screening

There was considerable variation in response to screening on stem explants from the 37 cvs. Based on the response they were classified into four groups (Table 2). In 12 cvs. (32.4%) there were 1.6 or more shoots per explant and hence classified as Group 1. There were 13 cvs. (35.1%) which produced less than 1.6 shoots per explant and had no callus so were classified as Group 2. In nine cvs. (24.3%), only callus was formed so this formed Group 3, while three cvs. (8.2%) were completely inert which formed Group 4.

Regeneration of Group 2

The response of Group 2 cvs. to BAP concentrations and a statistical analysis for the same is presented in Table 3. There was successful regeneration in XW

Response of explant to screening	Group	Media	Decision I	Result of decision I	Decision II	Result of decision II
>1.6 shoots (stem ex)	**1**	**1**	Ready for transformation	**Table 2**	-	-
<1.6 shoots (stem ex)	**2**	**2a&b**	To medium with lower and higher BAP than **medium 1**	Three out of the 12 cultivars tested produced 1.6 or more shoots, **Table 3**	Test leaf explant for cultivars that fail to regenerate on **media 2 a&b**	No cultivar was regenerated successfully
Only Callus (stem ex)	**3**	**3a&b**	Callus induced on **medium 1** and transferred to shoot induction media	Three out of the nine cultivars tested produced 1.6 or more shoots	Test leaf explant for cultivars that fail to regenerate on **media 3a&b**	Three out of the five cultivars tested produced 1.6 shoots or more, **Table 7**
No response (stem ex)	**4**	**4 a-i**	Test NAA and BAP in 9 different combinations, **Table 4**	One out of the three cultivars tested produced more than 1.6 shoots	Test leaf explant for cultivars that fail to regenerate on **media 4 a-i**	The only cultivar tested did produce 1.6 shoots, **Table 7**
Lot of shoot primordia only (leaf ex) on medium 5	**All inert cvs. of groups 2 - 4**	**5&6d**	Transfer to a **medium 6d**	Produced the maximum number of shoots in this combination, **Table 6**	-	**Table 7**

Figure 1. Flow chart of the mode of advancing of several regeneration protocols based on the stem explant (stem ex) response to screening and shift to leaf explants (leaf ex) for recalcitrant cultivars. The number of harvestable shoots are per explant obtained as an average value of four replications, with each replication consisting of 10 explants.

109, Sheba and Tigerrag on medium 2a (BAP concentration 0.1 mg l^{-1}). BAP at 0.1 mg l^{-1} concentration is highly significant ($p = <.001$), for seven cvs. over 1.0 mg l^{-1}, while for three cvs. over 2.0 mg l^{-1}. However for three cvs. 1.0 mg l^{-1} BAP concentration is still significant over 2.0 mg l^{-1}.

Regeneration of Groups 3 and 4

Three out of nine cvs. of Group 3 tested (Moneymaker, Tourmalin and Tr. 2) produced 1.6 or more harvestable shoots per explant. One out of the three cvs. of Group 4 (CBA 2053) produced 2.3 harvestable shoots per explant on medium 4a (Table 4) and the rest remained recalcitrant.

In Table 5 all the cultivars regenerated using stem explants with their respective media are summarised.

Leaf explants

Response of leaf explants of cv. 1581 to medium 5 produced only primordia like structures with no further response hence they were transferred to regeneration media (6 a–i, Table 6). The results were assessed 42 days after the initiation of the culture, using the number of harvestable shoots developed in each of the

Table 4. The nine different combinations of NAA and BAP tested along with MS salts and vitamins on stem explants of Group 4 cultivars

Media	NAA mg l^{-1}	BAP mg l^{-1}
4a	1	1
4b	1	2
4c	1	4
4d	2	1
4e	2	2
4f	2	4
4g	4	1
4h	4	2
4i	4	4

Table 5. Number of shoots regenerated using stem explants, of cultivars from the four different groups

Cultivar	Media	Group	No. of shoots	Cultivar	Media	Group	No. of shoots
Cleo	1	1	4.6	Tornato	1	1	1.6
Rush	1	1	3.6	Lineker	1	1	1.6
Kris	1	1	2.8	Tigerrag	2a	2	2.5
CBA 1403	1	1	2.6	Sheba	2a	21.6	
Albert Hein	1	1	2.3	Xw 109	2a	2	1.6
Toulouse	1	1	2.1	Tr. 2	3 a&b	3	2.5
Venezia	1	1	2.1	Tourmalin	3 a&b	3	1.6
Tr. 1	1	1	2.0	Money m	3 a&b	3	1.6
Mayfair	1	1	1.8	CBA 2053	4a	4	2.5
Biarritz	1	1	1.6				

Modifications tested for regenerating each group (media 2a, 3a, b and 4a). The number of harvestable shoots are per explant obtained as an average value of four replications, with each replication consisting of 10 explants.

treatment. Induction on medium 5 for four days was slightly better than eight, which was as good as 16. Four days of induction was best for explants regenerating on media numbers 6d, 6e and 6g. Eight days of induction did best on regenerating media 6f, 6h and 6i. The 16-day induction period did best on regeneration media 6a–c. However the highest number of harvestable microshoots was recorded for 4 days of induction followed by transfer to a regeneration medium 6d (0.0 mg l^{-1} IAA and 0.25 mg l^{-1} BAP, Table 6). Hence this combination (media 5 and 6d) was selected for testing of cvs. recalcitrant on stem explants.

Regeneration of cultivars recalcitrant on stem explant

The combination of media 5 and 6d was extended to 14 recalcitrant cvs. from Groups 2, 3 and 4. Successful regeneration was obtained in 4 of the 14 cvs. tested (Rainbow, TR-5, Hugo and Mike). Three other cvs., Poso Doble, TR-2 and Stallion, also responded, but produced less than 1.6 shoots per explant. The remaining seven cvs. produced five to eight primordia only without further development. The list of cvs. and their response is mentioned in Table 7. No cultivar from Group 2 was successfully regenerated using leaf explants.

Discussion

Using modifications of protocols used by Gutterson et al. (1994), De Jong et al. (1995) and Lemieux et al. (1991), we have developed a protocol flowchart (Figure 1) leading to regeneration in 23 of the 37 cvs. The modifications investigated were based on the response of the explants and not trial and error. The hormone concentrations ranged from equimolar amounts of auxin and cytokinin (Kaul et al., 1990) to the use of a 2 fold molar excess of cytokinin (Earle et al., 1974; Bush et al., 1976; Slusarkiewicz et al., 1981; Ledger et al., 1993; Lowe et al., 1993; Urban et al., 1994). Cultivar requirements in chrysanthemum for regeneration appeared quite stringent, which has been previously reported (Kaul et al., 1990; Sauvadet et al., 1990: Lowe et al., 1993).

Potential of varying the concentration of BAP to influence the shoot number is evident from the regeneration of Group 2 cvs. It is interesting to note that the reduction in BAP concentration in medium 2a induced a better response on three cultivars. Such responses in improved regeneration upon lowering of cytokinins has been observed in brassica (Block et al., 1989). It is possible the endogenous levels of cytokinins in these three cultivars are so high that upon placing on a media with low BAP the conditions suitable for regeneration are created. However, the reasons for differences in endogenous cytokinin levels and their differential response to regeneration is still unclear. We have efficient assays for measuring endogenous levels of cytokinin oxidases but not endogenous cytokinin levels. The low levels of cytokinins in addition make it more difficult to evolve and standardise such protocols.

Table 6. The influence of induction period (days on media 5) and media after induction for regeneration of leaf explants of Cv. 1581

Media number	Media BAP	× IAA	Induction period 4 days	8 days	16 days
6a	0.0 mg l^{-1}	0.0 mg l^{-1}	0.3	0.1	0.4
6b	0.0 mg l^{-1}	0.25 mg l^{-1}	0.8	0.1	0.4
6c	0.0 mg l^{-1}	0.50 mg l^{-1}	0.25	0.2	0.5
6d	0.25 mg l^{-1}	0.0 mg l^{-1}	2.5	2.1	1.6
6e	0.25 mg l^{-1}	0.25 mg l^{-1}	0.6	0.3	0.2
6f	0.25 mg l^{-1}	0.50 mg l^{-1}	0.3	0.4	0.3
6g	0.50 mg l^{-1}	0.0 mg l^{-1}	1.9	1.1	1.6
6h	0.50 mg l^{-1}	0.25 mg l^{-1}	0.5	0.5	0.4
6i	0.50 mg l^{-1}	0.50 mg l^{-1}	0.4	0.7	0.2
	Mean		0.7	0.6	0.6

Based on the data the protocol of 4 days induction on media 5 and regeneration on a media 6d was selected. The numbers of harvestable shoots are per explant obtained as an average value of four replications, with each replication consisting of 10 explants.
The variate is number of shoots with LSD at 1.7.

Table 7. Shoot regeneration from leaf explants of cultivars recalcitrant on stem explants (from Groups 2 to 4)

Varieties	Group	Shoots	Varieties	Group	Shoots	Varieties	Group	Shoots
Rainbow	4	1.6	TR-2	3	1.0	Roubiax	2	0
TR-5	3	1.7	Stallion	2	0.7	David	2	0
Hugo	3	1.6	Lemans	2	0	Granada	2	0
Mike	3	1.8	Alencon	2	0	TR-3	2	0
Paso doble	3	1.3	Cocarde	2	0			

Among the 14 cultivars tested, four showed successful regeneration when induced on medium 5 and regenerated on medium 6d. The number of harvestable shoots are per explant obtained as an average value of four replications, with each replication consisting of 10 explants.

Initially callusing stem explants on medium 1 and later transferring them to media 3 a,b provided a method to regenerate Group 3. The media 3 a,b composed of different vitamins and hormones could have induced differentiation in the calli. Different sources of cytokinin (BAP, coconut water and kinetin) could generate varied response like protein synthetic capacity, growth status (Gaudino and Pikaard, 1997) and thus influence organogenesis. The switch from BAP to kinetin and finally to a medium with GA3 did induce organogenesis in our calli. Kinetin is a non aromatic cytokinin as compared to BAP which may have had a different response on the explants. GA3 is a known agent in inducing organogenesis in calli, including long-term calli of citrus (Chakravarty and Goswami, 1999).

Stronger auxin (heat stable NAA) could induce better de-differentiation signifying their potential in inducing regeneration of otherwise recalcitrant explants. Group 4 failed to have any response in the screening on medium 1, but by providing a stronger auxin it was possible to regenerate some of them. Varying auxin source, concentration and time of exposure would be combinations worth testing on recalcitrant explants. Stronger auxins induce higher levels of polyamines (Maatar and Hunault, 1997) which could directly or indirectly affect the regeneration process.

The results on leaf explant based regeneration implied the role of hormones, especially auxin in regeneration. By pulsing high concentrations of auxin (medium 5) for different time periods and subsequently transferring to different hormone combinations, it was

possible to regenerate genotypes recalcitrant on stem explants. The cvs. from Groups 3 and 4 produced direct organogenesis on leaf explants upon strong auxin pulsing, which was not so for cultivars from Group 2. For a given genotype the effect of a certain set of hormone and media combinations may have varying influences based on the explant.

A methodology for developing regeneration protocols based on response of the explant, proved to be successful. Material recalcitrant for a given explant can be regenerated using alternate explants and media combinations. Regeneration could be further influenced by the quality of light incident on the cultures.

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