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Research Article

Factors Influencing *in Vitro* Organogenesis of *Chrysanthemum morifolium* cv. 'Resomee Splendid'

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Background: Chrysanthemum; also commonly known as mums or chrysanths, is one of the most important ornamental crops worldwide. Introducing desirable traits into this valuable plant by the conventional breeding has so far been faced with some restrictions due to the limited gene pool and cross-incompatibility. Therefore, breeders have decided to exploit *Agrobacterium*-mediated transformation methods in order to satisfy the growing market demands. However, more efficient *in vitro* regeneration protocols are required for this approach.
Objectives: The objective of this research was to develop an efficient protocol for an *in vitro* plant regeneration by the examining the effects of various combinations and concentrations of the plant growth regulators (PGRs) and different explants types.
Materials and Methods: The leaf and petiole explants of the *Chrysanthemum morifolium* cv. 'Resomee Splendid' were collected from the *in vitro* grown plantlets. Murashige and Skooge (MS) medium was supplemented with different concentrations and combinations of benzylaminopurine (BAP), 1-naphthaleneacetic acid (NAA) and thidiazuron (TDZ). Thereafter, the effects of these hormonal treatments were investigated on shoot initiation percentage, the average number of shoots per explants, callogenesis, and the type of organogenesis in regard to both types of the explants.
Results: Shoots were directly formed from leaf explants on the media that only contained BAP without callus formation. Amongst the other hormonal treatments, a combination of 4.5 $mg.L^{-1}$ BAP plus 1 $mg.L^{-1}$ NAA resulted in the direct organogenesis from the leaf explants, which was superior to the other combinations and concentrations. In regard to the petiole explants, direct shoot formation occurred in all the media except for the ones which were fortified with TDZ. In this case, considering the shoot initiation percentage and the mean shoot number per explants, the best results were achieved in the medium supplemented with 1.5 $mg.L^{-1}$ BAP and 1 $mg.L^{-1}$ NAA. Results showed that interaction of either BAP or TDZ with NAA was necessary for the callus induction.
Conclusions: Significant differences in shoot initiation percentage and the average number of shoots per explants were observed both in leaves and petioles grown on different media. Moreover, the callogenesis rates, as well as organogenesis types, showed some differences among the studied explants when compared on the same media.

Keywords: Callogenesis, *Chrysanthemum morifolium,* Explants types, Plant growth regulators, Regeneration

1. Background

Chrysanthemum which is widely known as "Autumn Queen" belongs to the Compositae (Asteraceae) family of plants (1). Due to the high popularity and demand, chrysanthemum is considered as one of the first commercial plants for micropropagation (2). Tissue culture studies on chrysanthemum were first started in 1952 by Morel and Martin (3). Adventitious *in vitro* shoot regeneration in chrysanthemum is the most commonly used technique for producing new cultivars, either in mutation breeding (4) or in genetic transformation (5, 6). Formation of the new organs is a distinctive characteristic of the plant regeneration (7). However, it is affected by the various factors such as composition of the medium, plant growth regulators (PGRs) interaction, type of explants, and plant genotype

(8,9, 10). In tissue culture, the usage of PGRs play a crucial role in different plant processes consisting mostly of the growth, differentiation, and development; for instance, shoot initiation, callogenesis, embryogenesis, and rooting (11). The presence of auxin in the marked combinations with cytokinins in the medium is essential to obtain adventitious shoot induction (12). However, the concentration of the applied growth regulators is an important factor. Moreover, the interactions of the different PGRs are very important, as well (13). The various endogenous phytohormone's levels of the different tissue explants might affect the requirements for the exogenous PGRs (14). Effect of plant growth regulators on *in vitro* shoot regeneration from leaf explants of the chrysanthemum was studied in an investigation. The maximum percentages of the shoot regeneration in addition to shoots *per se* per explants in the studied cultivar were achieved on a medium containing equal concentrations (0.5 $mg.L^{-1}$) of the NAA and BA (15). Also, The impact of various types of explants on regeneration has been studied in prior investigations, such as petal (5, 16, 17), leaf (17, 18), stem (17), and petiole (6, 17). The efficiency of the leaf, shoot tip, and ray floret explants of the chrysanthemum for the direct *in vitro* regeneration were compared in a survey and shoot tips were reported to be the most suitable types of explants (19). To emphasize more, it should be acknowledged that different PGRs show dissimilar peculiarity and requirements for these hormones differs amongst various explants (20). Furthermore, protocols suitable for a given cultivar are not necessarily efficient for the others (17).

2. Objectives

The objective of the present attempt was evolving an efficient plant regeneration protocol via organogenesis from the leaf and petiole explants treated with different concentrations of various PGRs, as part of our further effort to develop a sustainable transformation system for *Chrysanthemum morifolium* cv. 'Resomee Splendid'.

3. Materials and Methods

3.1. Plant Material, Media Preparation, and Growth Conditions

A constant supply of the plant material for conducting the experiment was obtained by cutting the nodal segments from the four-month-old donor plants of *Chrysanthemum morifolium* cv. 'Resomee Splendid'. After discarding the leaves, the collected material was brought to the laboratory and washed thoroughly with running tap water for 30 min. Then the explants were dipped in 70% ethanol for 30 s, followed by immersion in a 2% sodium hypochlorite solution containing a droplet of Tween 20 in constant agitation for 10 min. The nodal cuttings were finally washed thrice with sterilized distilled water for 20 min. Afterward, the explants were cultured in MS (21) medium supplemented with 0.25 $mg.L^{-1}$ BAP. The medium contained 30 $g.L^{-1}$ sucrose and was solidified with 6.5 $g.L^{-1}$ plant agar. The pH was adjusted to 5.8 before autoclaving at 121 °C for 15 min. All cultures were exposed to the fluorescent lights with a 16/8h light/dark photoperiod at 25±2 °C. Eventually, the explants were obtained from *in vitro*-grown 7-weeks-old plantlets. Excluding the distal and basal portions of the lamina, leaves were cut into 5 mm^2 square explants with a mid-rib portion. Petioles were also cut into *ca*. 0.3-0.5 cm long sections. All the explants were horizontally placed on the medium with the adaxial wounded surface down. Regeneration was induced on MS media supplemented with the different concentrations of the either BAP or TDZ in combination with NAA as shown in **Table 1**. Thereafter, the regenerated adventitious shoots were separated from the initial explants and were transferred to a hormone-free MS medium in which elongation, as well as rooting, were occurred. The individual rooted plantlets, of about 5-6 cm in length, were taken out from the jars and washed carefully with running tap water in order to remove the agar. Afterward, plantlets were transferred to the plastic cups filled with sterilized Cocopeat and Perlite (a mixture of 1:1 ratio of each) and were covered with the same plastic material as a lid. During a week all the plantlets were successfully acclimatized and transplanted into soil.

3.2. Experimental Design and Statistical Analysis

A completely randomized design (CRD) experiment was laid out with two explants types and 16 different growth regulator treatments, consisting of the three replicates with five explants per each. The data were recorded every two weeks for different parameters including shoot initiation percentage, average number of shoots per explant, and callus induction. The final experimental observations were recorded which after 7 weeks of the culture were statistically analyzed using SPSS software, analysis of the variance (ANOVA), Afterwards, means were compared by Duncan's multiple range test.

4. Results

4.1. Shoot Initiation on Leaf and Petiole Explants

In the present study, shoot formation was initiated within 2-3 weeks both in the case of leaf and petiole explants

Table 1. The effect of the two main factors including explant types and PGR treatments on the shoot initiation percentage and the mean shoot number per explant in *Chrysanthemum morifolium* cv. 'Resomee Splendid' measured 7 weeks after culture.

Main factor	Shoot initiation (%)	Mean shoot number/explant
Explant type		
Leaf	45.00 a*	1.07 a
Petiole	41.66 a	1.08 a
PGR treatment (mg.L^{-1})		
T1= 0.0 BAP + 0.0 NAA	0.00	0.00
T2= 1.5 BAP + 0.0 NAA	3.33 b	0.10 d
T3= 3.0 BAP + 0.0 NAA	10.00 b	0.23 cd
T4= 4.5 BAP + 0.0 NAA	20.00 b	0.93 bc
T5= 0.0 BAP + 0.5 NAA	0.00	0.00
T6= 1.5 BAP + 0.5 NAA	56.66 a	1.03 bc
T7= 3.0 BAP + 0.5 NAA	66.66 a	1.90 a
T8= 4.5 BAP + 0.5 NAA	63.33 a	1.93 a
T9= 0.0 BAP + 1.0 NAA	0.00	0.00
T10= 1.5 BAP + 1.0 NAA	83.33 a	2.06 a
T11= 3.0 BAP + 1.0 NAA	63.33 a	1.36 ab
T12= 4.5 BAP + 1.0 NAA	80.00 a	2.06 a
T13= 0.0 TDZ + 0.1 NAA	0.00	0.00
T14= 0.4 TDZ + 0.1NAA	30.00 b	0.53 cd
T15= 0.6 TDZ + 0.1NAA	23.33 b	0.33 cd
T16= 0.8 TDZ + 0.1NAA	20.00 b	0.50 cd

* Means with the same letters within an individual main factor are not significantly different at $p < 0.05$.

regardless of the hormonal treatments. New shoots most intensively appeared at cut sites between the 4th and the 7th week of explant culture on the medium.

We noticed that shoot initiation was dependent on the explant type. As presented in **Table 2**, using leaf explants, shoot regeneration occurred in T2 (1.5 mg.L^{-1} BAP), T3 (3 mg.L^{-1} BAP), and T4 (4.5 mg.L^{-1} BAP) (**Fig. 1**A) treatments with a significant improvement in the both quantitative parameters keeping pace with the increased concentrations of the BAP (**Table 3**). whereas, petiole explants were gradually turned brown and deteriorated on the same media.

4.2. Direct and Indirect Organogenesis

As delineated in Table 2, although regenerated shoots in T6 (1.5 mg.L^{-1} BAP+ 0.5 mg.L^{-1} NAA), T7 (3 mg.L^{-1} BAP + 0.5 mg.L^{-1} NAA), T8 (4.5 mg.L^{-1} BAP + 0.5 mg.L^{-1} NAA), T10 (1.5 mg.L^{-1} BAP+ 1 mg.L^{-1} NAA)

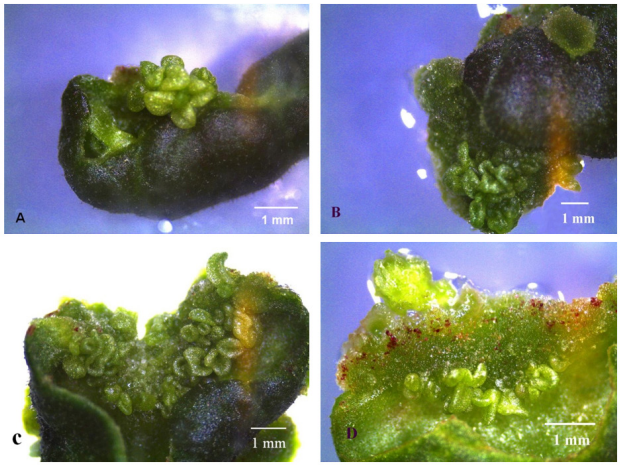

Figure 1. Adventitious shoot regeneration from the leaf explants in *Chrysanthemum morifolium* cv. 'Resomee Splendid' 17 days after culturing in the different media. **A:** Direct organogenesis without any callus formation in the T4 medium (4.5 mg.L^{-1} BAP + 0.0 mg.L^{-1} NAA), **B:** An indirect organogenesis from the callus in the T10 medium (1.5 mg.L^{-1} BAP + 1.0 mg.L^{-1} NAA),**C:** Direct organogenesis in T12 medium(4.5 mg.L^{-1} BAP + 1.0 mg.L^{-1} NAA) despite of the callus formation, **D:** Indirect organogenesis from anthocianin-enriched callus in the T16 (0.8 mg.L^{-1} TDZ + 0.1 mg.L^{-1} NAA).

Table 2. The effect of PGR treatments and explant types on shoot initiation percentage, average number of shoots per explant and callogenesis in *Chrysanthemum morifolium* cv. 'Resomee Splendid' measured 7 weeks after culture.

PGR/ treatment (mg.l^{-1})	Leaf			Petiole			Remarks	
	Shoot initiation (%)	Mean shoot number/ explant	Callogenesis	Shoot initiation (%)	Mean shoot number/ explant	Callogenesis	Leaf	Petiole
T1	0.00	0.00	-	0.00	0.00	-	Necrosis	Necrosis
T2	6.66 d*	0.20 c	-	0.00	0.00	-	D	Necrosis
T3	20.00 cd	0.46 bc	-	0.00	0.00	-	D	Necrosis
T4	40.00 abcd	1.86 ab	-	0.00	0.00	-	D	Necrosis
T5	0.00	0.00	-	0.00	0.00	-	Rooting	Rooting
T6	60.00 abc	1.06 abc	+	5.33 bc	1.00 cd	+	ID	D
T7	53.33 abc	1.20 abc	++	80.00 ab	2.60 a	+	ID	D
T8	66.66 abc	1.53 abc	++	60.00 ab	2.33 ab	++	ID	D
T9	0.00	0.00	-	0.00	0.00	-	Rooting	Rooting
T10	73.333 ab	1.33 abc	++	93.33 a	2.80 a	++	ID	D
T11	46.66 abcd	1.13 abc	++	80.00 ab	1.60 bc	+++	ID	D
T12	80.00 a	2.00 a	+++	80.00 ab	2.13 ab	+++	D	D
T13	0.00	0.00	+	0.00	0.00	-	Rooting	Rooting
T14	40.00 abcd	0.80 abc	+	20.00 cd	0.26 d	+++	ID	ID
T15	26.66 bcd	0.46 bc	+	20.00 cd	0.20 d	+++	ID	ID
T16	26.66bcd	0.86 abc	++	13.33 d	0.13 d	+++	ID	ID

(*) Means within a column followed by different letters are significantly different according to Duncan's Multiple Range Test at $p < 0.05$.
(-) No callus formation, (+) weak callus formation, (++) moderate callus formation, (+++) good callus formation, (D) Direct organogenesis, (ID) Indirect organogenesis.

(Fig. 1B), and T11(3 mg.L^{-1} BAP+ 1 mg.L^{-1} NAA) were formed indirectly from leaves, surprisingly, direct organogenesis occurred on the aforementioned media from petiole explants (**Fig. 2**). In T12 (4.5 mg.L^{-1} BAP+1 mg.L^{-1} NAA), despite of good callus formation, direct regeneration was observed in both cases (Fig. 1C) (Table 2).

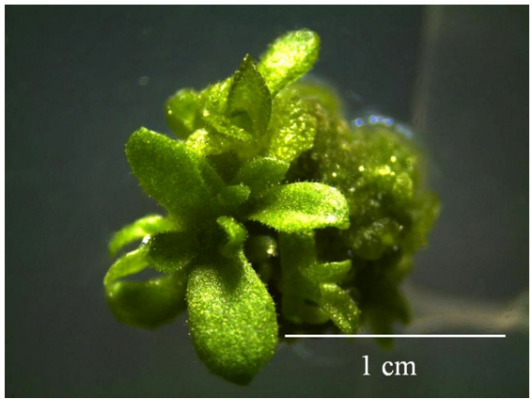

Figure 2. Direct shoot formation from the petiole explants in *Chrysanthemum morifolium* cv. 'Resomee Splendid' 4 weeks after culturing in the T10 medium (1.5 mg.L^{-1} BAP + 1.0 mg.L^{-1} NAA).

4.3. Callogenesis

Taking callogenesis from leaf explants into consideration, results showed a poor callus formation with fairly green color in the T14 (0.4 mg.L^{-1} TDZ+ 0.1 mg.L^{-1} NAA) and T15 (0.6 mg.L^{-1} TDZ+0.1 mg.L^{-1} NAA). whereas, anthocyanin-enriched callus in some petiole explants were formed in these media as well as in the T16 (0.8 mg.L^{-1} TDZ + 0.1 mg.L^{-1} NAA) with a good callusing rate (Fig. 1D) (Table 2). Moreover, using leaf explants, anthocyanin-enriched calli were also recognized in T6 (1.5 mg.L^{-1}BAP+ 0.5 mg.L^{-1} NAA), and T7 (3 mg.L^{-1} BAP+ 0.5 mg.L^{-1} NAA). Another remarkable observation in both explant types was related to the indirect shoot induction and bigger size of the regenerants in T14, T15, and T16 (**Fig. 3**) compared to the other treatments (Table 2).

4.4. Plant Growth Regulator Treatments

As shown in Table 1, plant growth regulator treatments were significantly different. Data presented in Table 2 for the leaf explants illustrated the superiority of the T12 (4.5 mg.L^{-1} BAP+ 1 mg.L^{-1} NAA) over all the other treatments in regard to both shoot initiation percentage (80%) and the average number of shoots per explant (2.00). On the other hand, using petiole explants, the

Table 3. The effect of various concentrations of BAP on shoot initiation percentage, the mean shoot number per explant and callogenesis in *Chrysanthemum morifolium* cv. 'Resomee Splendid' measured 7 weeks after culture.

BAP concentration (mg.L^{-1})	Shoot initiation (%)	Mean shoot number per explant
T1= 0	0.00 b*	0.00 b
T2= 1.5	6.66 b	0.20 b
T3= 3	20.00 ab	0.46 b
T4= 4.5	40.00 a	1.86 a

* Means within a column followed by different letters are significantly different according to Duncan's Multiple Range Test at $p < 0.05$.

best results were achieved in T10 (1.5 mg.L^{-1} BAP + 1 mg.L^{-1} NAA) with 93.33% and 1.80 for shoot initiation percentage and the average number of shoots per explant, respectively. Intriguingly, our data also revealed that in the absence of auxins in T2 (1.5 mg.L^{-1} BAP), T3 (3 mg.L^{-1} BAP), and T4 (4.5 mg.L^{-1} BAP) treatments, direct organogenesis was favored without an intervening callus phase. whereas, shoot initiation was inhibited and roots were formed without callus formation in the T5 (0.5 mg.L^{-1} NAA), T9 (1 mg.L^{-1} NAA), and T13 (0.1 mg.L^{-1} NAA) which were only supplemented with the different concentrations of NAA.

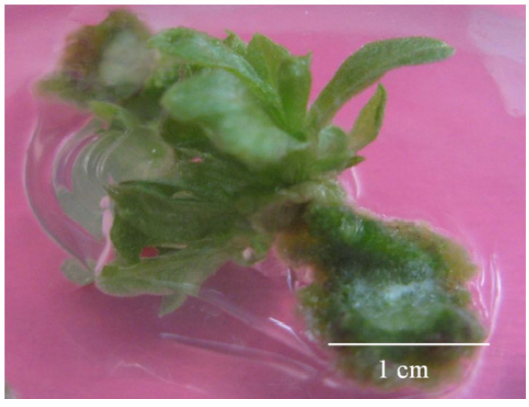

Figure 3. Indirect shoot formation from petiole explants in *Chrysanthemum morifolium* cv. 'Resomee Splendid' 4 weeks after culture in T16 (0.8 mg.L^{-1} TDZ + 0.1 mg.L^{-1} NAA). Note the remarkable size of the regenerants after this period.

5. Discussion

Plants start the process of regeneration through at least two different cellular strategies which include reactivation of the relatively undifferentiated cells and reprogramming of the differentiated somatic cells. Totally, regeneration depends on the cellular plasticity, which can be defined as the ability to re-specify the cell fate (7). Plant regeneration is usually affected by the genotype, PGRs, and explants types, though plant cell's totipotency theoretically enables each cell to keep the ability to regenerate the whole new plant through somatic embryogenesis or organogenesis (17, 22). Several premier requirements for a successful micropropagation protocol include simple media formulation, minimum production stages and consistent rates of propagation (23). Lee *et al*. (24) have observed that the greatest regeneration of the adventitious shoots on the leaf disk explants in *Chrysanthemum coronarium* L. occurs after the 5th week of the culture. whereas, the leaf primordial formation from the callus has already begun after 3 weeks of the culture. In another research on *Chrysanthemum grandiflorum* (Ramat.) Kitam. 'Satinbleu', the greatest dynamic of the regeneration on leaves was observed between the 3rd and the 5th week of culture (18). Considering that regeneration mostly started at the cut sites, it can be concluded that wound stimuli might supply a primary inductive trigger for the mentioned phenomenon (7, 25, 26). Several cellular reactions such as production of the plant hormones are induced by wounding (7).

A number of previous reports have confirmed that BAP accelerates the development of the bud initials, causing the increased number of the bud's primordial in the chrysanthemum (27, 28). It has also been claimed that BA is often effective when added separately to the medium for an *in vitro* culture (29). Song *et al*. (17) have reported a relatively feeble adventitious shoot induction on the medium supplemented with the BA alone. Waseem *et al*. (30) have used different concentrations of the BAP (0, 0.5, 1, and 2 mg.L^{-1}) and have noticed that by an increase in the BAP concentration the rate of regeneration from the shoot tip explants was reduced in chrysanthemum, which is in conflict with our results on the leaf and petiole explants. Kaul *et al.* (31) have reported that various parts of the plant respond differently to the PGRs added to the medium, which is in coincidence with our observations considering different reactions of the petioles and leaves. A possible explanation could be that BAP concentrations or

individual application of this PGR were not appropriate for the shoot induction from the petiole explants. Another reason might be that the petiole cells have less organogenic potential compared to the leaf explants as it was proposed by Lim *et al.* (6) to explain the better regeneration from stem explants compared to the leaves and petioles. The ability to synthesize and react to cytokinins naturally differs. Therefore, increasing the applied cytokinin levels is useless when tissues are not responsive to this phytohormone (32).

The effect of explants source on direct shoot organogenesis has been reported before (17, 19, 32). Such variations can be caused by age (33) and physiological condition of the explants, and are likely to be influenced by the genetic factors (34). Furthermore, genotype remains a key determinant of the direct shoot organogenesis in chrysanthemum (6). Kaul *et al*. (31) reported that direct shoot regeneration from leaf or petiole explants has advantages over the shoot induction from an initial callus phase which may result in a somaclonal variation and chimerism.

The choice of cytokinin can affect regeneration type (17). It has been claimed that TDZ promotes callus production rather than shoot regeneration in *Lilium longiflorum* (36) which is in coincidence with our results, especially in regard to petioles (Table 2). On the other hand, TDZ is also known to induce multiple shoots in a broad range of plant species (37, 38, 39). In regard to the size of the regenerants, it seems that TDZ as a cytokinin, not only has affected cell division but also has greatly promoted cell enlargement even in low concentrations.

Growth regulators are an integral part of all *in vitro* investigations (40). The regenerative capacity of the plant cells *in vitro* can be promoted by culturing explants on media supplemented with PGRs (32). Considering that cytokinins and auxins are mutually dependent, several physiological effects of the cytokinins can be described by their interaction with the auxins (17). Chadhury and Rongda (41) have also shown that exogenous requirements of the PGRs for the induction of an organogenic reaction depend on the defined concentrations of the endogenous PGRs. Moreover, the most appropriate ratio between cytokinin and auxin for shoot organogenesis in chrysanthemum is conflicting among investigators (42). Hence, by manipulating the compositions and concentrations of the growth regulators in the medium, the regeneration frequency can be enhanced.

Nahid *et al*. (5) have explained that BA alone or combined with the kinetin had no effect on callus growth. On the other hand, Fuji and Shimizu (43) have reported that no callus was induced when the petal and achene explants of the *Chrysanthemum coccineum* were cultured on BA-free medium. Thereby, it is suggested that BA combined with NAA may be critical for callus induction of chrysanthemum (5, 44). Waseem *et al*. have reported the regeneration of chrysanthemum plantlets from leaf disk (43) and shoot tip explants (30) with an application of 0.5, 1 or 1.5 $mg.L^{-1}$ NAA alone which is in conflict with our results. This might be due to the fact that NAA usually does not respond well against to shoot proliferation (45) and is known for its root formation ability (46). Naing *et al.* (47) have reported that leaf explants of the chrysanthemum cv. Vivid Scarlet were more responsive to NAA than BAP, while our results emphasize on a greater role of BAP on the regeneration. These observations can reflect the various endogenous hormone levels already present in different tissues which might affect responsiveness to the exogenously applied hormones (31, 48, 49).

Song *et al.* (17) have studied the efficiency of shoot regeneration from leaf and petiole explants of the chrysanthemum using different combinations and concentrations of BA, IAA, and kinetin. Based on their results, among three cultivars, the minimum and maximum of the mean number of shoots formed per leaf explants were 0.0 and 0.29, respectively, in different PGR treatments. While in our experiment we obtained 0.0- 2.0 shoots per leaf explants (Table 2). Song *et al*. (17) have also reported 0-19% shoot induction (%) from the leaf explants of these cultivars; while, we achieved 0-80% shoot initiation in the different media (**Table 2**). In regard to the petiole explants, Song *et al* (17) have reported that the mean number of shoots formed per explant were between 0-0.05 and shoot induction percentage was 0-4.8% for three cultivars in different media, whereas, our shoot number per petiole explants was 0-2.8 and shoot initiation (%) was 0- 93.3%. Lim *et al*. (6) showed that among 11 chrysanthemum cultivars, only one cultivar regenerated shoots from leaf and petiole explants (66.67% and 23.33%, respectively) on MS medium solidified with agar and supplemented with 1 μM NAA and 10 μM BA.

Finally, it should be re-mentioned that plant regeneration is usually affected by the genotype, PGRs and the explants types (17, 22). Hence, each of these factors can influence the efficiency of a proposed protocol.

6. Conclusion

Shoot regeneration from each type of the explants of *Chrysanthemum morifolium* cv. 'Resomee Splendid' was optimized by testing the effects of various

combinations and concentrations of PGRs. The shoot initiation percentage and the average number of regenerated shoots per explants differed significantly among various hormonal treatments. Taking leaf and petiole explants into consideration, different responses were obtained in each PGR treatment in regard to each of the investigated characteristics. We have successfully established a useful system for an *in vitro* regeneration of the *chrysanthemum morifolium* cv. 'Resomee Splendid' via organogenesis which can further facilitate the genetic transformation of this cultivar.

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