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Naphthaleneacetic Acid Solely Induced Extensive Hairy Root Development that Precedes Extensive Shoot Regeneration from Stalk Explants of Cauliflower (*Brassica Oleracea* Var. Botrytis)

Hani Al-Ahmad*[1]

[1]*Department of Biology and Biotechnology, An-Najah National University, Nablus, Palestine.*

**Corresponding author:* *Dr. Hani Al- Ahmad, Department of Biology and Biotechnology, An-Najah National University, Nablus, Palestine, Tel: +972-9-234-5113; Fax: +972-9-234-5982; E-mail: alahmad@najah.edu*

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Abstract

A reliable, rapid, and single step strategy for *in vitro* regeneration of true-to-type cauliflower (*Brassica oleracea* var. Botrytis) plants is described. Mature stalk disc explants cultured on Murashige and Skoog (MS) medium supplemented with 2 mg l^{-1} naphthaleneacetic acid (NAA) regenerated entire plants within one month. During the first 18 days, hairy roots developed intensively before the regeneration of shoots (on average 52 shoots per 1-cm disc explant) on the same MS medium plus 2 mg l^{-1} NAA. Stem elongation and full plantlet development were successfully achieved within 10 days on MS medium without plant growth regulators, followed by plantlets transplantation into soil. This rapid and large scale propagation protocol was faster, easier and showed higher frequency of shoot induction comparing to the routine shoot-then-root organogenesis protocols using various combinations of cytokinins. On the other hand, NAA solely induced callus and hairy roots, but with poor shoot regeneration from cauliflower floret curd tissues compared to that from stalk disc explants.

Keywords: Cauliflower; Organogenesis; Stalk; Floret Curd; One Step Cloning Protocol

Abbreviations

BAP: 6-benzylaminopurine; 2,4-D: 2,4-dichlorophenoxyacetic acid; GA_3: Gibberellic Acid; IBA: Indole-3-butyric acid; Kinetin (= KIN), 6-furfurylaminopurine; MSO: MS Medium Devoid of Plant Growth Regulators; NAA: Naphthaleneacetic Acid; PGRs: Plant Growth Regulators

Introduction

Cauliflower (*Brassica oleracea* var. botrytis) is an economically important vegetable crop worldwide. A rapid and efficient method for cauliflower *in vitro* micropropagation from explant to whole plant is required for regeneration of purebred lines for conventional breeding programs, and for crop improvement using modern genetic transformation routes, as well [1,2]. A variety of explants have been used for regeneration of cauliflower, including seedling cotyledons, hypocotyls, true leaves, floral tissues [1-9], and zygotic embryos [10]. However, the use of stem explants was limited to Bhalla and Weerd [2]. In general, authors used different types of floral and vegetative tissues as explants including 2-3mm stem pieces for initial shoot regeneration under the influence of the cytokinin 6-benzylaminopurine (BAP). Thus, the inductive capacity of auxins as naphthaleneacetic acid (NAA) and other cytokinins as kinetin for shoot regeneration was examined in this study using larger stalk (stem) disc explants.

Typically, with conventional nursery practice, cauliflower plants can be propagated within a month after seed germination. Thus, a rapid *in vitro* cloning protocol is required for commercial competitive application purpose. In addition to cauliflower genotypes and the source of explants, the combinations of growth regulators used showed differential response. For example, Bhalla and Weerd [2] found that shoot induction from leaf-derived explants required the presence of both cytokinin and auxin in the culture media. However, they found that the cytokinin BAP alone was enough for shoot induction from floral-derived tissues. These results differ from earlier study reported by Kumar et al., [4] who regenerated plants from floret curd explants in one-step and rapid method using indole-3- acetic acid (IAA) alone. This kind of one-month approach using

vegetative tissues including stalk has not been tested yet, thus, in this study, it was tested under the influence of NAA. In addition, the effect of the kinetin (as cytokinin) on shoot induction from cauliflower floret curds has also been investigated in this study.

In this paper, a rapid, reproducible, and efficient single step method for complete plantlet regeneration of cauliflower in almost a month is described. Moreover, the effects of various hormonal compositions on the rapidity of plantlets regeneration from stalk and floret tissues are also reported.

Materials and methods

Plant material and explants preparation

Cauliflower main stalks (stems) were obtained from commercial field-grown mature winter plants (Candid Charm hybrid), which formed full white floret heads. After removing the leaves, the stalks were cut into about 4cm in depth and sterilized with 70% ethanol for 2min, followed with 10% sodium hypochlorite for 15 min, and lastly washed 3 times with sterile distilled water. The stalk explants were prepared using sterile cork borer (1cm in diameter) that was pushed vertically through a stalk piece and the internal tissue was cut into individual discs of about 3mm in depth, which were immediately cultured on plant culture media as described below.

Direct organogenesis from cauliflower stalk disc explants

In vitro direct regeneration of whole cauliflower plants from sterilized stalk disc explants was induced on medium containing Murashige and Skoog (MS) [11] salts with vitamins (Duchefa Biochemie, Netherlands), and supplemented with 3% (w/v) sucrose, 100 mg l^{-1} myoinositol (Sigma, USA), and 0.55% (w/v) phytagel (Sigma, USA) as a solidifying agent. The pH of the MS medium was adjusted to 5.8 before autoclaving at 121°C and 15 psi pressure for 20min. The MS basal medium was supplemented with different plant growth regulators (PGRs) including 2 mg l^{-1} NAA, or 2 mg l^{-1} BAP plus 1 mg l^{-1} NAA, or 3 mg l^{-1} BAP plus 0.5 mg l^{-1} gibberellic acid (GA_3), or 5 mg l^{-1} 6-furfurylaminopurine (kinetin = KIN), 5 mg l^{-1} kinetin plus 0.5 mg l^{-1} GA_3, and MS medium free of PGRs (MSO) as control. All treatments were internally replicated, and repeated twice using stalks from different healthy donor mother cauliflower plants. All cultured plates were incubated under controlled environment of 25 ± 2°C, 16h white light of 80 µmoles m^{-2} sec^{-1} and 8h of darkness to stimulate adventitious shoot development, and were transferred into fresh media periodically every 3 weeks, if needed. The frequency of shoot regeneration and mean number of shoots per stalk disc explant were determined after 4 weeks. Developed shoots were transferred into MSO for stem elongation, then to MS containing 1 mg l^{-1} indole-3-butyric acid (IBA) if failed to form roots on the initial culture medium or on MSO.

Since various levels of regeneration efficiency have been obtained in previous studies that used different shoot tissues from different cultivars of cauliflower (for example: Bhalla and Weerd [2]); floret curd tissues were also examined in this study for comparison with the stalk disc explants just described. Small white floret tissues were sterilized as above, and then cultured on MS medium supplemented with 2 mg l^{-1} NAA, or 5 mg l^{-1} kinetin, 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), and MSO as PGRs free medium control.

All plant growth regulators used in this paper were supplied from Sigma, St. Louis, MO, USA, and their optimal concentrations were based on our preliminary experiments.

Plantlets transplantation and acclimatization

For plant hardening and maturation, well established plantlets were removed from the culture medium and the roots were washed gently with tap water to remove the remains of agar medium. The plantlets were then transplanted in 1-L pots containing soil and vermiculite in 1:1 ratio, covered with plastic bags to maintain high humidity, and transferred to growth chamber that maintained 25 ± 2°C and 16h white light of 80 µmoles m^{-2} sec^{-1} and 8h of darkness for one week for hardening. The potted plants were then transferred to ambient laboratory environment and the plastic bags were removed gradually during another week. Finally, the plants were grown to maturity under glasshouse conditions.

Statistical analysis

The shoot regeneration parameters were presented as the average ± standard error (SE), and analyzed using the Minitab 17® software by one-way analysis of variance (ANOVA) and by comparing the Fisher's least significant differences (LSD). Probability levels were considered to be statistically significant at $P \leq 0.05$ and highly significant at $P \leq 0.01$. Differences were not considered to be statistically significant at $P > 0.05$.

Results and discussion

Naphthaleneacetic acid induced efficient micropropagation of cauliflower plants from stalk explants

When white mature cauliflower stalk disc explants were cultured for two weeks on medium containing MS salts and vitamins, and supplemented with 3% (w/v) sucrose, 100 mg l^{-1} myo-inositol, and solely 2 mg l^{-1} NAA, short and viable white hairy roots developed intensively around the periphery of explants and from the bottom of greenish stalk discs (Figure. 1A and B). This was immediately followed

by extensive shoot regeneration from the discs periphery maintained on the same MS medium plus 2 mg l^{-1} NAA (Figure. 1C). The primordial leaves were purple then became green upon maturation by the 18th day after cultures initiation (on average 52 shoots per 1-cm disc explant) (Figure. 1D). Individual stalk discs were divided into several sectors; each with many roots but with limited number of less than 1-cm in size purple shoots, and transferred to glass jars containing MS medium devoid of PGRs (MSO) for stem elongation and entire plantlet formation. Within 10 days on MSO, all fully regenerated plantlets were ready for transplantation into soil (Figure. 1E). Thus, the entire micropropagation procedure required almost four weeks for the regeneration of huge number of cauliflower plants; starting from stalk disc explants cultured on one kind of medium. Approximately, 96% of the regenerated plantlets survived after transfer to the glasshouse (Figure. 1F).

These results are consistent with similar developmental patterns of extensive production of hairy roots before the regeneration of shoots, achieved with cauliflower protoplast-derived callus cultured in the presence of NAA and BAP [12]. A rapid single step regeneration method was also reported by Kumar et al., [4] who used cauliflower floral curd explants and regenerated high frequency of plants in the presence of low concentration of IAA alone.

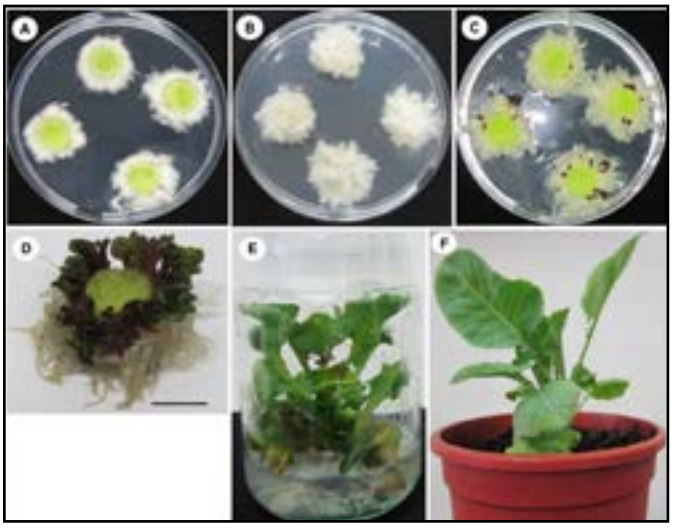

Figure 1. Rapid and large scale cauliflower plantlets regeneration from mature stalk explants induced by naphthaleneacetic acid applied solely. Hairy roots developed within 2 weeks on stalk disc explants cultured on MS medium plus 2 mg l^{-1} NAA, top view (A) and bottom view (B). Immediate shoot induction from rooted stalk explants on MS medium plus 2 mg l^{-1} NAA (C). A single stalk disc developed large number of shoots took from MS medium plus 2 mg l^{-1} NAA culture after 18 days (scale bar 1.0cm) (D). Fully developed plantlet is ready for transplantation into soil, after one month from culture initiation (E). True-to-type regenerated cauliflower plant grown in greenhouse environment (F).

In this study, intensive root differentiation did not show negative effects on the subsequent shoot regeneration, since all rooted stalk discs regenerated high frequency of viable shoots without hyperhydricity. In addition, this method is faster, less laborious and more effective comparing to other micropropagation techniques including indirect organogenesis; where callus should be developed at first, or the ordinary shoot-then-root direct organogenesis; where difficulties in the root induction on developed shoots may arise (Table 1).

The use of mature stalk explants for *in vitro* propagation of cauliflower requires plants to be grown to the curd stage before propagating. In contrast, advantages of using explants from vegetative tissues include the year-round availability. Additionally, many authors reported the efficient use of seedling cotyledonary or hypocotyle explants [1,5,6,8,9], as well as the zygotic embryos [10] for regeneration of different cauliflower genotypes. However, sterile plants may arise during conventional breeding and modern genetic engineering programs (for example; [2,13]) therefore, development of vegetative propagation method(s) based on explants such as stalk discs is required to ensure the cloning of such sterile lines.

In conclusion, following this one-step protocol, it is feasible to reduce the *in vitro* clonal propagation time into 4 weeks; rather than several weeks with other shoot-then-root based techniques [14], and as reported below.

Influence of cytokinin-auxin combinations on cauliflower regeneration from stalk discs

In this part of the current study, various frequencies of relatively long term shoot-root regeneration were obtained from stalk disc explants cultured in the presence of different combinations of BAP, NAA, GA_3, and kinetin (Table 1). The number of shoots developed per stalk explant relatively increased after the 6th week of cultures initiation, however, the shoot induction frequency at the 4th week on medium containing 2 mg l^{-1} NAA was still superior compared to those on cytokinin supplemented medium ($P \leq 0.001$) (Table 1). Among the four combinations containing cytokinins, the best medium for shoot induction was the one containing 3 mg l^{-1} BAP plus 0.5 mg l^{-1} GA_3. In this case, the rate of responsive explants reached 98%, and the average number of shoots per disc explant was 15, when measured after 4 weeks (Table 1). However, on average, up to 21 shoots per explant were observed after 6 weeks of cultures initiation, without root regeneration (Figure. 2A). Roots developed within 2 more weeks; when the initial shoots were transferred into MSO or medium with 1 mg l^{-1} IBA for elongation and root development (Figure. 2B and C). This is almost double the time interval required to reach the stage of plant transfer to field environment following the regeneration induced on stalk discs on medium with 2 mg l^{-1} NAA in the one-step rapid cloning protocol; described above.

Table 1. Frequency of shoot regeneration from stalk disc explants of cauliflower (*Brassica oleracea* var. Botrytis).

Plant growth regulator (mg l^{-1})	Induction features	Induction onset (week)	Mean number of shoots per disc of 4 weeks cultures	Induction rate (%)
MSO	White stalk discs turned green	1st	0.00 ± 0.00[d]	___
2 NAA	**Extensive hairy roots before intensive shoot development. No callus induction**	**2nd for shoots**	**52.00 ± 2.14[a]**	**100**
2 BAP+1 NAA	Few shoots with little callus and few hairy roots	4th	4.30 ± 0.42[c]	93
3 BAP+0.5 GA_3	**Greenish discs with shoots**	**3th**	**15.20 ± 1.44 [b]**	**98**
5 KIN	Greenish discs with shoots	4th	4.88 ± 0.52[c]	90
5 KIN+0.5 GA_3	Greenish discs with shoots	4th	7.40 ± 0.54[c]	95

Each treatment consists of two replications and in each replication 25-30 stalk disc explants were used.
Means that do not share a letter are significantly different based on LSD values at P ≤ 0.05. N = 10-15.

Other PGRs combinations were also used in this study under the same environmental and culture conditions, but with lower rate of shoot induction than that of the 3 mg l^{-1} BAP plus 0.5 mg l^{-1} GA_3, just described. Noticeably, kinetin influence on shoot induction from cauliflower stem tissues was not investigated yet. Thus, it was included in this investigation. Although ≥ 90% of the cultured stalk discs responded to exogenous cytokinins, the numbers of developed shoots were extremely low compared to that of stalk cultures initiated on 2 mg l^{-1} NAA medium (< 8 shoots per explant vs. 52 shoots per explant, respectively) (P ≤ 0.01). In contrast, there was no significant difference in the number of shoots developed from stalk explants cultured in the presence of 5 mg l^{-1} kinetin alone or 5 mg l^{-1} kinetin plus 0.5 mg l^{-1} GA_3 or 2 mg l^{-1} BAP plus 1 mg l^{-1} NAA (Table 1).

The presence of NAA in the last combination induced little calli and few hairy roots at the expense of shoot production. This is consistent with Bhalla and Weerd [2] observations, where BAP induced shoot regeneration from 2-3mm stem explants of commercial (B-4) hybrid of autumn cauliflower. Addition of 0.2 mg l^{-1} NAA to the former media reduced the rate of shoot development [2].

Influence of naphthaleneacetic acid on cauliflower regeneration from floret curd tissues

In addition to the effect of genotype of cauliflower on regeneration, the source of explants and the combinations of growth regulators added to the medium can also induce different responses [2, 4].

Table 2. Induction potential of floret curd explants of cauliflower (*Brassica oleracea* var. Botrytis) cultured on various PGRs.

Plant growth regulator (mg l^{-1})	Induction features	Induction rate (%)	Fig. No.
MSO	Greenish explants without callus or shoots. Long white roots developed in 3 weeks	80	3F
1 (2,4-D)	-White callus developed at curd cut sites during the first 2 weeks	55	___
	-Flower buds turned purple, become elongated and converted into narrow-blade leaflets after the 4th week	35	3D
2 NAA	-Hairy roots and callus developed in 2-3 weeks	100	3B
	-Few leaflets regenerated from purple elongated flower buds during the 4th-6th weeks	80	3C
5 KIN	-Intensive shoots developed from cut curd sides within 3 weeks	100	___
	-Within 5-6 weeks, some flower buds elongated, opened and were purple, but did not convert into true leaves	___	3E

Each treatment consists of two replications and in each replication 15-20 floret curd explants were used.

Hence, to evaluate the regeneration capacity of the cauliflower commercial cultivar used in this study, plant regeneration using floral tissues was also tested in a comparison experiment with the stalk disc explants. Axenic white floret curd pieces (Figure 3A) were cultured on MS medium supplemented with 2 mg l^{-1} NAA alone, as well as with 1 mg l^{-1} 2,4-D alone and 5 mg l^{-1} kinetin alone (Table 2).

On the contrary to the rapid root-then-shoot regeneration from stalk disc explants due to NAA described above, it revealed that efficient shoot regeneration from floret tissues requires exogenous cytokinin. Within 3 weeks, hairy roots plus callus were regenerated around the cut sites of almost all the curd tissues cultured on medium included 2 mg l^{-1} NAA (Table 2, Figure. 3B). During the 4th week, the flower buds of most cultures turned purple, elongated and developed few narrow leaf blades with long petioles (Figure. 3C), but no shoots developed from the curd itself in the presence of 2 mg l^{-1} NAA. In contrast, Kumar et al., [4] reported efficient shoot induction from floret tissues under the influence of IAA alone, but they observed stunted shoots in the presence of IAA with BAP.

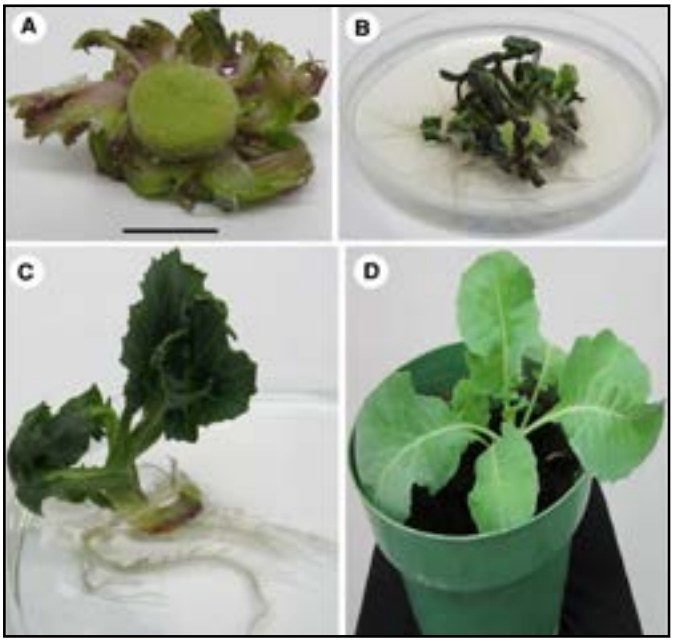

Figure 2. Shoot-then-root regeneration from cauliflower stalk disc explants cultured on MS medium containing 3 mg l^{-1} BAP plus 0.5 mg l^{-1} GA_3. Shoots regenerated within 6 weeks (scale bar 1.0 cm) (A). Shoots developed roots after transfer into MSO plus 1 mg l^{-1} IBA (B). Complete plantlet ready for transplantation into soil after 9 weeks of propagation initiation (C). Regenerated cauliflower plant grown in the greenhouse (D).

On the contrary to the rapid root-then-shoot regeneration from stalk disc explants due to NAA described above, it revealed that efficient shoot regeneration from floret tissues requires exogenous cytokinin. Within 3 weeks, hairy roots plus callus were regenerated around the cut sites of almost all the curd tissues cultured on medium included 2 mg l^{-1} NAA (Table 2, Figure 3B). During the 4th week, the flower buds of most cultures turned purple, elongated and developed few narrow leaf blades with long petioles (Figure 3C), but no shoots developed from the curd itself in the presence of 2 mg l^{-1} NAA. In contrast, Kumar et al., [4] reported efficient shoot induction from floret tissues under the influence of IAA alone, but they observed stunted shoots in the presence of IAA with BAP.

When similar floret explants were cultured in the presence of 1 mg l^{-1} 2,4-D alone, white-yellowish calli developed at curd cut sites of the explants in the rate of 55% within 2 weeks. Some flower buds became purple, elongated, and developed into limited number of narrow-blade leaflets af--ter the 4th week of cultures initiation (Table 2, Figure 3D). This was a common response by the two auxins used in this study, the NAA and the 2,4-D.

When white floret curd tissues were cultured on MS medium containing 5 mg l^{-1} kinetin for 3 weeks; huge numbers of purple leaf tissues were regenerated from the curd cut sides of all cultured floret explants (Table 2, Figure 3E). Shoots continued normal development during the 4th week.

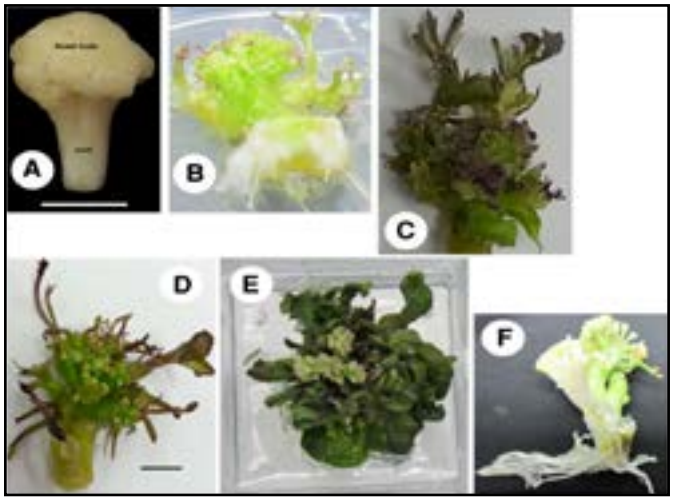

Figure 3. Naphthaleneacetic acid induced callus and hairy roots with poor shoot regeneration from cauliflower floret curd tissues. Axenic white floret curd pieces used for *in vitro* micropropagation (scale bar 1.0cm) (A). Roots and calli regenerated within 3 weeks from the cut sites of the curd tissues cultured on MS medium including 2 mg l^{-1} NAA (B). Conversion of some elongated flower buds into leaflets within 4 weeks on MS medium including 2 mg l^{-1} NAA (C). Some purple and elongated flower buds converted into few leaflets after the 4 weeks on MS medium including 1 mg l^{-1} 2, 4-D (scale bar 1.0cm) (D). Extensive shoot regeneration from curd explants cultured on MS medium containing 5 mg l^{-1} kinetin for 4 weeks (E). Rooting of floret curd explants cultured on MSO for 3 weeks (F).

These results are consistent with that of floral-derived tissues tested by Bhalla and Weerd [2] under the induction of BAP, but again differ from the earlier reported by Kumar et al., [4], who regenerated plants from curd explants on IAA containing medium. This difference in tissue culture

cauliflower used. During the 5th-6th weeks, some flower buds became elongated, opened and almost purple, but did not developed into true leaves (Table 2).

On the other hand, almost 80% of the plant cultures on MSO developed white long roots within 3 weeks of cultures initiation, indicating that initial shoots can be transferred into MSO medium for simultaneous shoot elongation and rooting, without including IBA (Table 2, Figure 3F). Thus, shoots obtained from the 5 mg l^{-1} kinetin cultures were rooted on MSO and MS plus 1 mg l^{-1} IBA as well, and then transferred to soil under glasshouse conditions as described in the "Materials and methods" section. Approximately 95% of the transplanted plantlets survived and grew normally to maturity.

In conclusion, for applying genetic engineering in plant improvement programs, and for propagating a male sterile line or cloning a purebred line, both stem and floret curd plant tissues can be used as explant for efficient micropropagation process, taking the advantage of the one step and rapid 2 mg l^{-1} NAA regeneration protocol reported herein.

Acknowledgments

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