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Callus Induction on True Shallot Seed Explant Using a Combination of BA and 2,4-D

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ABSTRACT

BA and 2,4-D combination were commonly used for in vitro culture of Shallot (*Allium cepa* L. var agregatum 2n = 2x = 16) to induce callus, but there was no information for callus induction on shallot seed (TSS) explant. Callus could be utilized for in vitro selection and generating of genetic variation. The aims of the research was to identify the response of TSS (Trisula and Tuk Tuk) as explant and to obtain the optimum combination of BA and 2,4-D ($mg.L^{-1}$): (0–0, 2–1, 2–2, 2–3, and 2–4) in callus induction. The research had been carried out in the Tissue Culture Laboratory, Faculty of Agriculture, Universitas Gadjah Mada during the year 2015-2016. Factorial treatments of variety and growth regulators were arranged in Completely Randomized Design with four replications. Data of percentage of germination, shoot height, root length, percentage of callus formation, callus weight, and chromosomes number of callus were recorded. The results showed that combination of 2 $mg.L^{-1}$ BA + (1– 4) $mg.L^{-1}$ 2,4-D induced callus formation on TSS but inhibits shoots and roots growth. The best callus proliferation was at a concentration of 1 $mg.L^{-1}$ 2,4-D. Tetraploid callus chromosomes (2n = 4x = 32) was detected in Trisula grown in the 2 $mg.L^{-1}$ BA + 4 $mg.L^{-1}$ 2,4-D, but in the Tuk Tuk callus did not detected the changing of chromosomes number.

Keyword: Chromosome number, plant growth regulator, tetraploid

INTRODUCTION

Shallot (*Allium cepa* L. var agregatum2n =16) is an important vegetable crop in Indonesia. Commonly, shallot is propagated using a bulbs. Propagation using bulbs produces low shallot variation. This problem can be solved by the development of genetic variation through callus induction. Callus can be utilized for in vitro selection and production of genetic variation (Chauhan and Kothari, 2004). Callus is an unorganized mass of plant cell. Callus growth is influenced by several factors i.e. genotype, explant source, and plant growth regulator. Different genomes will have different responses to the given induction (Leva *et al.*, 2012). Different source of explant is considered as the most critical variable for resulting different frequency of the frequency and trait of the callus. Callus is controlled by growth-regulatingsubstances present in the medium (auxin and cytokinins) (Shah *et al.*, 2003).

Flower bud (Sulistyaningsih *et al.*, 2006) and bulbs of shallot (Hailekidan *et al.*, 2013)could produce callus when cultured in vitro, but there is no information about seedas source of explant.Shallot seed called botanical seed or true shallot seed (TSS). TSS structure consists of seed coat, endosperm, and embryo. Embryo consists of plumule and radicle (Triharyanto and Purnomo, 2014). Sharma *et al.* (2005) reported that the embryo was often used as explant source because it has high potential of regeneration. TSS contained mature embryos andendosperms. Itiseasy to be sterilized, so itcanbe used as an source of explant in the production callus of shallot.

Growth regulators as part of the culture medium have an essential role in tissue culture, specifically in controlling biological processes in plant tissues. Auxin and cytokininscan induce callus formation (Leva *et al.*, 2012) and unstable chromosomes. The most commonly auxin used for callus induction is 2,4-D (2,4-Dichlorophenoxyacetic acid). During the callus phase,sometime it caused change of the chromosomes number. The callus sometimes may alter their

chromosome number such as polyploid cell or aberration chromosome cellas reported by Niizeki and Zhongen (2003). 2,4-D may cause abnormal spindle structure in mitotic division (Duval *et al.*, 1998).BA (6-Benzyl Adenine)induced formation of compact, embryogenic structure of callus (Chaudhury and Qu, 2000).

The aims of the research was to identify the response of TSS (Trisula and Tuk Tuk) as explant and to obtain the optimum combination of BA and 2,4-D ($mg.L^{-1}$) in callus induction.

MATERIALS AND METHODS

The research work had been carried out at Tissue Culture Laboratory, Faculty of Agriculture, Universitas Gadjah Mada during the year 2015–2016. All research were setup in the completely randomized design factorial (2 varieties and 5 concentration of 2,4-D) with 4 replicates. Each replicate was represented by one jar containing 10 TSS explants.

Explant material

The seed (TSS of *Allium cepa* L. group Aggregatum) of two varieties, namely Trisula and Tuk Tuk were obtained from BPTP Central Java and East West Seed Indonesia. Seeds were washed using tap water and liquid soap in order to clean some contaminants from the seed. Seeds were soaked in 70% alcohol for 2 min, then sterilized with sterile water containing one drops of tween and 5% commercial bleach for 3 min. Seeds were rinsed several times with sterile distilled water.

Medium for callus induction and regeneration

Seeds were cultured in a solid agar medium containing the mineral salts of Murashige and Skoog (1962), 30 $g.L^{-1}$ sucrose, and 5 combination of BA and 2,4-D (0–0, 2–1, 2–2, 2–3, 2–4) $mg.L^{-1}$. pH medium was adjusted to 5.8 and then added with 7 $g.L^{-1}$agar. Medium were autoclaved at 121ºC for 20 min. The explants were cultured and incubated at growth room, with 23 ± 2 ºC and under light for 16/8 light/dark period during 30 days.All explants(callus and planlet) generated then transferred to jars containing a regeneration medium that contained half strength of MS basic medium. The jars were incubated in growth room during 45 days.

Chromosome detection

Pre-treatment of callus and rootwere in thesterile water for 24 hours at temperature 4 ºC and then fixation carried out in the solution of glacial acetic acid and alcohol (1:3) for 24 hours.Hydrolysiswas done using 1 N HCl at 60 ºC for 10 minutes. After hydrolysis, the materials were transferred directly to fuchsinthen were squashed to produce chromosome preparation. Chromosome preparation were observed under microscope.

Statistical analysis

Observations were consisted of the growth and development of explants during incubation includes percentage of germination, shoot height, root length, percentage of callus formation, callus weight, and number of callus chromosomes. Data were analyzed using SAS for ANOVA and Duncan's multiple range test (DMRT) was applied at $P \leq 0.05$.

RESULT AND DISCUSSION

Trisula and Tuk Tuk varieties showed flowering ability and produced seeds that can be used as a source of TSS explants. TSS seeds are black in color and rounded flat. Normal TSS showed complete structure of embryo, endosperm, and testa that will grow into normal plants (Triharyanto and Purnomo, 2014). Initial response to TSS growth on the medium of callusinduction was germination. In this research, the highest percentage of germination was 72.50 % (Figure 1). There was

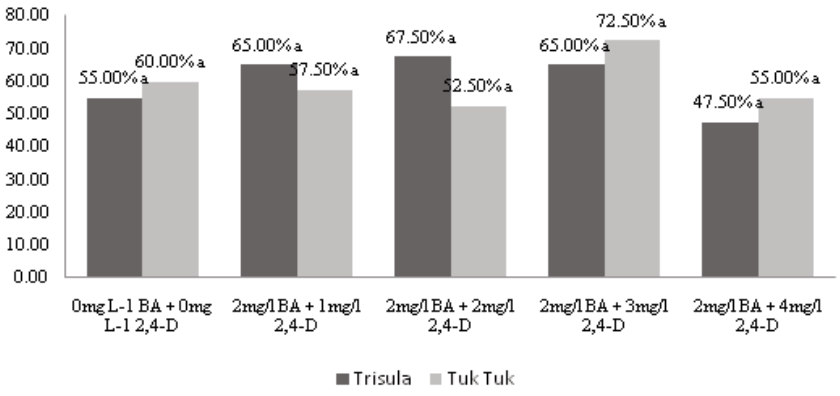


Figure 1. Effect of different variety and medium on percentage of germination

Table 1. Effects of different variety and medium on shoot height (cm) and root length (cm) of TSS 30 days after induction

Medium	Shoot height (cm)		Mean	Root length (cm)		Mean
	Trisula	Tuk Tuk		Trisula	Tuk Tuk	
0 mg.L^{-1} BA + 0 mg.L^{-1} 2,4-D	9.50	9.13	9.31 a	4.00	3.38	3.69 a
2 mg.L^{-1} BA + 1 mg.L^{-1} 2,4-D	3.50	2.63	3.06 b	0.45	0.50	0.48 b
2 mg.L^{-1} BA + 2 mg.L^{-1} 2,4-D	4.15	2.11	3.13 b	0.53	0.50	0.51 b
2 mg.L^{-1} BA + 3 mg.L^{-1} 2,4-D	2.75	2.63	2.69 b	0.53	0.50	0.51 b
2 mg.L^{-1} BA + 4 mg.L^{-1} 2,4-D	3.00	2.75	2.88 b	0.50	0.50	0.50 b
Mean	4.58 a	3.85 b	(-)	1.20 a	1.08 a	(-)
CV (%)	23.04			18.89		

Remarks: Remark: The means in the column or row followed by the same letter were not significantly different according to DMRT (α 5%); (+) = interaction; CV= coefficient variation.

Table 2. Effects of different variety and medium on percentage of callus formation (%) and callus weight (g) 75 days after induction

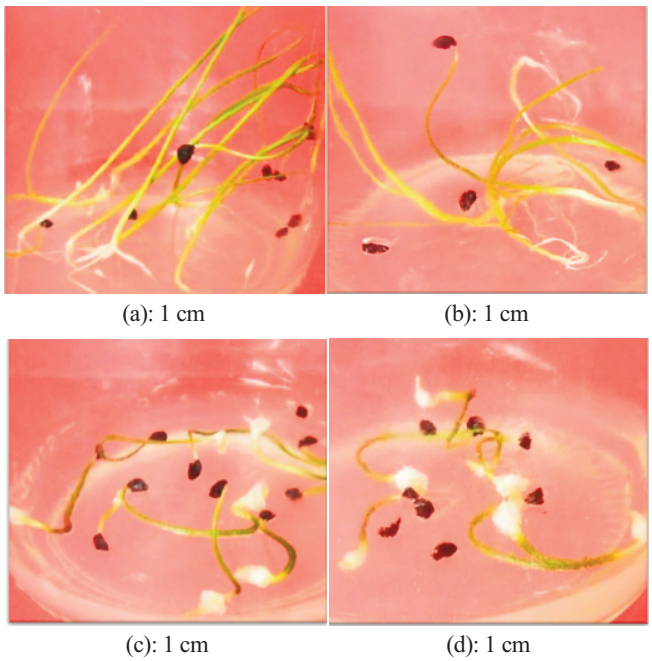
Medium	Shoot height (cm)		Mean	Root length (cm)		Mean
	Trisula	Tuk Tuk		Trisula	Tuk Tuk	
0 mg.L^{-1} BA + 0 mg.L^{-1} 2,4-D	0.00	0.00	0.00 b	0.00	0.00	0.00 b
2 mg.L^{-1} BA + 1 mg.L^{-1} 2,4-D	65.00	57.50	61.25 a	2.16	0.89	1.53 a
2 mg.L^{-1} BA + 2 mg.L^{-1} 2,4-D	67.50	52.50	60.00 a	0.91	0.43	0.67 b
2 mg.L^{-1} BA + 3 mg.L^{-1} 2,4-D	65.00	72.50	68.75 a	0.54	0.89	0.71 b
2 mg.L^{-1} BA + 4 mg.L^{-1} 2,4-D	47.50	55.00	51.25 a	0.88	0.68	0.78 ab
Mean	49.00 a	47.50 a	(-)	0.90 a	0.58 a	(-)
CV (%)	43.27			50.54		

Remarks: Remark: The means in the column or row followed by the same letter were not significantly different according to DMRT (α 5%); (+) = interaction; CV= coefficient variation.

no significant difference in the percentage of germination towards the treatment of varieties and media.

There was no significant difference ininteraction effects of variety and concentration of 2,4-D on shoot height, root length, percentage of callus formation and callus weight (Table 1 and 2). The variation of germination could be determined from the growth difference between control and BA and 2,4-D effect in shoot height and root length. There was also a noticeable difference of high shoots on different varieties and media. Trisula shoot was higher (4.58 cm) than Tuk Tuk (3.85 cm). It implied that the sprout growth was influenced by plant genetic. The medium showed the result with the highest shoot and the longest root of culture was the control (0 mg.L^{-1} BA + 0 mg.L^{-1} 2,4-D) resulting 9.31and 3.69 cm respectively, while 4 other treatments of media with combination of BA and 2,4-D showed no difference of shoot height and root length (Table 1). This suggests that the treatment of 2 mg.L^{-1} BA combined with 1–4 mg.L^{-1} 2,4-D may inhibit the growth of shoots and roots. Application of growth regulators at high concentrations could delay cell division activity and disrupt cell metabolism that inhibits plant growth (Salisbury and Ross, 1995). The variation of morphology could be determined from the growth characteristics of TSS during culture. In the control medium, shoots and roots grew normally, whereas in the medium with the addition of 2 mg.L^{-1} BA + (1–4) mg.L^{-1} 2,4-D growth of shoots and roots was inhibited andswelling response on the white stem (Figure 2). The swelling stem is the part that develops into callus.

Auxins are used to induce callus and root while cytokines are used to induce shoots (Jouanneau, 1971). BA induces the formation of adventive shoots more effective, while 2,4-D plays a role in inducing callus (Flick *et al.*, 1993). In this research, the addition of 2 mg.L^{-1} BA + (1–4) mg.L^{-1} 2,4-D showed the response of callus formation (47.50–72.50)%, while the control of 0 mg.L^{-1} BA and 0 mg.L^{-1} 2,4-D showed no callus for mationin both cultivars,Trisula or Tuk Tuk varieties (Table 2). Callus initiation started from 20 days after incubation. A higher cytokinin (BA) ratio of auxin (2,4-D) also showed response to callus formation. This means that the addition of exogenous 1–4 mg.L^{-1} 2,4-D induces the formation of TSS callus. Previous research conducted by Hailekidan *et al.* (2013) also mentioned that



(a): 1 cm (b): 1 cm

(c): 1 cm (d): 1 cm

Figure 2. Effect medium on TSS response (a) Trisula 0 mg L^{-1} BA + 0 mg L^{-1} 2,4-D (b) Tuk Tuk 0 mg L^{-1} BA + 0 mg L^{-1} 2,4-D (c) Trisula 2 mg L^{-1} BA + (1-4) mg L^{-1} 2,4-D (d) Tuk Tuk 2 mg L^{-1} BA + (1-4) mg L^{-1} 2,4-D

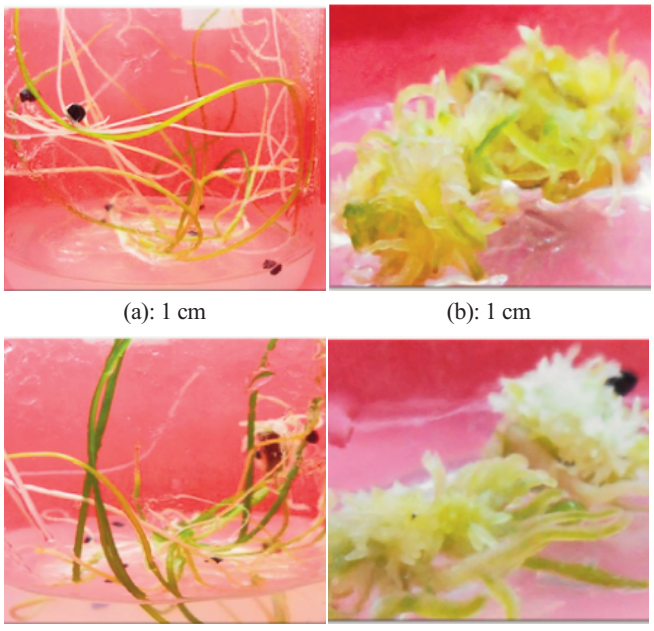


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Figure 3. Effect medium on TSS response Trisula (a) 0 mg.L^{-1} BA + 0 mg.L^{-1} 2,4-D (b) 2 mg.L^{-1} BA + (1-4) mg.L^{-1} 2,4-D and Tuk Tuk (c) 0 mg.L^{-1} BA + 0 mg.L^{-1} 2,4-D (d) 2 mg.L^{-1} BA + (1-4) mg.L^{-1} 2,4-D

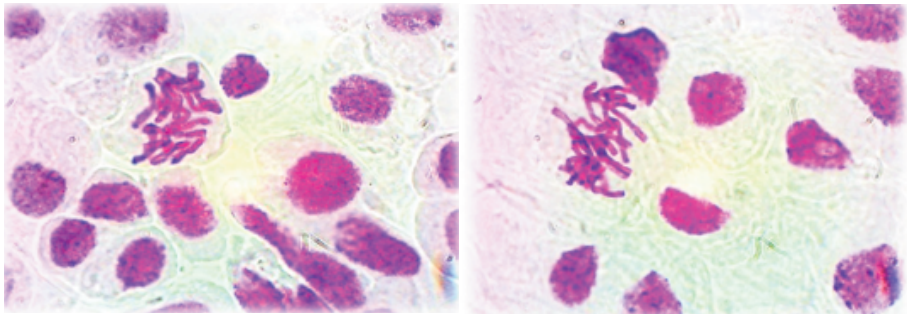
MS medium with 2,4-D addition showed the highest callus induction on shallot (74.44%).

After 30 days, calli were cultured on callus regeneration medium for 45 days. The half strength of MS medium without any plant growth regulator was failed to induce shoot or root. Explants cultured on the control medium at 0 mg.L^{-1} BA and 0 mg.L^{-1} 2,4-D treatment showed normal growth of shoots and roots, whereas explants cultured on the callus induction medium at 2 BA mg.L^{-1} and (1 - 4) mg.L^{-1} 2,4-D showed the positive growth of callus indicated by increasing callus size (Figure 3). Embryogenic callus had the ability to continue the proliferation to produce new callus cells and some of them were embryogenic in which callus could develop into somatic embryo (Kikuchi *et al.*, 2006). The callus types that were identified in this research were

homogeneous. Embryogenic callus showed characteristics, i.e. dry, crumb and yellowish white in color. In spite of the same callus characteristics, the callus weight indicates any differences among induction treatment media. Treatment of 2 mg.L^{-1} BA + 1 mg.L^{-1} 2,4-D showed the highest callus weight (1.53 g) (Table 2). This means that the best callus proliferation was at a concentration of 1 mg.L^{-1} 2,4-D.

One approach in the formation of variation is through callus. Callus allow for increased genetic variation wich considerable contribution to the plant breeding program. The mechanism of variation in tissue culture was poorly understood. Some of the possible mechanisms leading tovariation include karyotype changes, cryptic changes associated with chromosome rearrangement, transposable elements, somatic gene rearrangements, gene amplification and depletion, and somatic crossing over, sister chromatid exchange, and cryptic virus elimination (Larkin and Scowcroft, 1981). A karyotype change is one mechanism of variation that is easily observed due to the effect of growth regulator through changes in the number of chromosomes. The application of growth regulators on the induction of various causes chromosomal damage which ultimately lead to the changes in the number of chromosomes (Kaeppler *et al.*, 2000).

The cytological of callus were studied at 75 days after induction. Changes in chromosomal karyotype are commonly found in tissue culture. Attention was focused on chromosome number. This may cause changes in the number of chromosomes to polyploid or aneuploidy (Bhojwani and Dantu, 2013). Auxins (2,4-D) are often associated with genetic abnormalities such as polyploidy and stimulation of DNA synthesis that can lead to endoreduplication (Skirvin et al., 1993). Endoreduplication itself leads to the disruption of heterochromatin replication, cell cycle, and metabolism such as nucleotide imbalance. Aneuploids (monosomy



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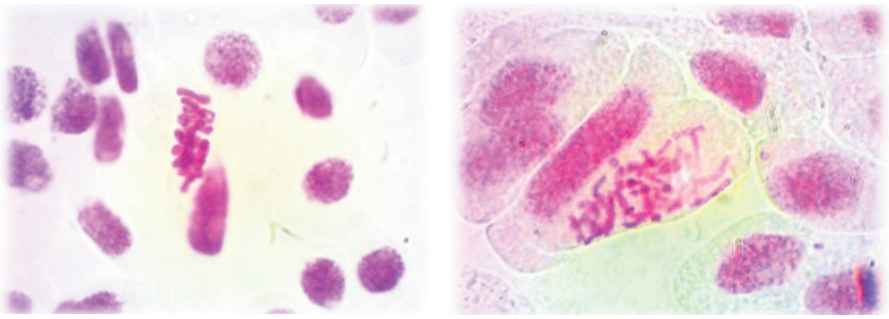
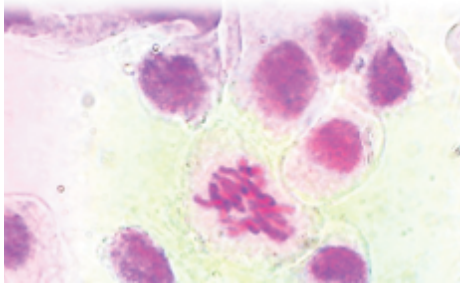


(c): 10 µm (d): 10 µm



(e): 10 µm

Figure 4. Chromosome number Trisula(a) control plant (diploid 2n = 16) (b) 2 mg.L^{-1} BA + 1 mg.L^{-1} 2,4-D (diploid 2n = 16) (c) 2 mg.L^{-1} BA + 2 mg.L^{-1}2,4-D (diploid 2n = 16) (d) 2 mg.L^{-1}BA + 4 mg.L^{-1} 2,4-D (tetraploid 2n = 32) (e) Tuk Tuk 2 mg.L^{-1} BA + 3 mg.L^{-1} 2,4-D (diploid 2n = 16)

and trisomy) are usually caused by nondisjunction occurrence that leads to chromosomal damage (Kaeppler *et al.*, 2000).

The chromosomes number in control treatment is observed through the root of explant, while the chromosomes number with 2,4-D treatments were observed through the callus of explant. Variation of chromosome number occurs in TSS callus. The chromosome number of control plants in Trisula was 2n = 16, but not all callus can be observed the number of its chromosomes. From the treatment of varieties and induction media, the change in callus chromosome number only occurred at Trisula 2 mg.L^{-1} BA + 4 mg.L^{-1} 2,4-D, while the callus in the other treatments remained diploid. The number of chromosomes changes from diploid (2n = 2x = 16) to tetraploid (2n = 4x = 32). Tuk Tuk callus does not result in a change in the number of chromosomes. The chromosome stability of plant callus depend on plant species (Niizeki and Zhongen, 2003). Some genomes can be stable when compared to other genomes (Leva *et al.*, 2012). Duval *et al.*, (1988) reported that 2,4-D causes spindle threads to be abnormal in mitotic division that can lead to diversity. Induction of exogenous growth regulators 2 mg.L^{-1} BA + 4 mg.L^{-1} 2,4-D for 30 days on culture media can stimulate cell division into polyploidy occurrence in Trisula varieties.The chromosome number observation of the callus tissue consist of 90% diploid, and 10% tetraploid cells. The variation of chromosome number occurs spontaneously and uncontrolled. This result in accordance with Niizeki and Zhongen (2003) result that cytological observation of the callus tissue consist of 22% haploid, 22% diploid, and 6% tetraploid cells after being induced with plant growth regulator.

In the regeneration of callus, explants showed no development of Shoots organs or even roots. However, the callus formed was embryogenic callus. Embryogenic callus has the regeneration ability in plant organs development (Sukmadjaja and Ade, 2008) such as shoots, leaves, and roots. This is in line to the previous research reported by Sulistyaningsih *et al.* (2006) and indicated that a combination of 2 mg.L^{-1} BA and 2 mg.L^{-1} 2,4-D 2 caused somaclonal variation in flower bud culture of shallot by producing trisomic and tetraploid plants from explants through callus. Thus, it is expected that the combination of 2 mg.L^{-1} BA + 4 mg.L^{-1} 2,4-D can also produce the Trisula tetraploid plantlet from the initial explant through the callus that can be a source of new genetic variation.

CONCLUSIONS

Combination of 2 mg.L^{-1} BA + (1-4) mg.L^{-1} 2,4-D inhibited shoots and roots growth but induces callus formation on TSS culture.The best callus proliferation was at a concentration of 1 mg.L^{-1} 2,4-D. The change number of Trisula callus chromosomes to tetraploid (2n = 4x = 32) in the 2 mg.L^{-1} BA + 4 mg.L^{-1} 2,4-D treatment indicated variation, whereas the Tuk Tuk callus did not result in a change number of chromosomes.

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