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Regular Article

Roles of plant growth substance in callus induction of *Achyranthes bidentate*

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In this research, callus from leaves, petioles and stems of *Achyranthes bidentata* was evidently initiated by plant growth substance, in which 2,4-dichlorophenoxyacetic acid (2,4-D) was very important to callus induction, but effects of other plant growth substances were various, and the optimum combination of plant growth substances for callus induction from leaves, petioles and stems was respectively obtained. Compared with callus induction from leaves and petioles, callus induction from stems was easier, and the higher induction rate and bigger mass of callus from stems were obtained. This study showed that the dedifferentiation capacity of various explants from *Achyranthes bidentata* was obviously different, and effects of plant growth substance on callus induction from various explants of *Achyranthes bidentata* were significantly diverse.

Keywords: *Achyranthes bidentata*, plant tissue culture, plant growth substance, callus.

Achyranthes bidentata is perennial herb, has many effects such as nourishing liver and kidney, strengthening sinews and bones, falling blood pressure, invigorating blood circulation silt, analgesia, etc., and so has been used in many prescriptions (Zhao *et al*., 2004). Recently the rapid study [illegible] *Achyranthes bidentata* has been made, plant tissue culture of *Achyranthes bidentata* has also been reported (Dong *et al*., 2002; Li *et al*., 2005), but the regeneration frequency of most explants is low, and sometimes appears obviously diverse to the same explants in different studies.

As is well known, the growth and development of plant need water, mineral and organic matter, and also are regulated by plant growth substance which is very critical to growth and differentiation of cell and tissue, induction and formation of bud and root (Melissa *et al*., 2006; Randy *et al*., 2009; Duan *et al*., 2013; Dragicevic *et al*., [illegible] At present, [illegible] cid (2,4-D), Naphthylacetic acid (NAA), 6-benzylaminopurine (6-BA) and other plant growth substances are often applied in plant tissue culture, in which NAA, 2,4-D and Indole butyric acid (IBA) belong to auxin and mainly play a role in callus formation, embryoid regeneration, and so on, yet 6-BA, Kinetin (KT) and Zeatin (ZT)

belong to cytokinin, and principally act on stimulants of cell division and induction of bud differentiation. Furthermore, the existence of synergistic, antagonistic or additive interaction between auxin and cytokinin has been demonstrated (Nordström *et al*., 2004; Aloni *et al*., 2006; López *et al*., 2007), and the varying ratios of cytokinin and auxin could induce plant cell to form particular tissue including callus, shoot, root or one whole plant (Melissa *et al*., 2006; Cui *et al*., 2010).

In this research, effects of plant growth substances such as 6-BA, 2,4-D, NAA, KT, IBA and ZT on callus induction from leaves, petioles and stems of *Achyranthes bidentata* were studied in order to establish the efficient system of plant regeneration for *Achyranthes bidentata* and provide theory basis for plant tissue culture and genetics breeding of *Achyranthes bidentata*.

Materials and Methods

Plant materials

Seeds of *Achyranthes bidentata* were kindly provided by Jiaozuo Academy of Agricultural Sciences, Henan, P.R. China. After seeds were soaked at 24℃ for 12h, surface-sterilized for 30s with 75% ethanol, subsequently were deep sterilized for 6min with 0.1% mercury bichloride, and then were washed five times with sterile water. The aseptic seeds were cultured on MS medium at 26±1℃ under 14h photoperiod of 1000-1200lux illumination intensity.

Experimental design

In this study, MS medium was used as basal medium, different concentration of 6-BA, 2,4-D, KT, NAA, IBA or ZT was respectively added into MS medium to explore effects of plant growth substance on callus induction from explants of *Achyranthes bidentata*. The orthogonal design of L_{18} (3^7) was adopted, kinds and concentrations of plant growth substances were listed in Table 1. On the basis of orthogonal array, 18 kinds of culture media supplemented with plant growth substances were designed to carry through callus induction, each culture medium consisted of 3.0% sugar, 0.7% agar and different kinds and concentrations of plant growth substances, besides the pH value of culture medium was adjusted to 7.0 or so.

Table 1: The Kind and concentration of plant growth substance in orthogonal array of L_{18}（3^7）

Factor / Level (mg/L)	6-BA	2,4-D	KT	NAA	IBA	ZT
1	0	0.5	0	0.5	0.1	0.1
2	0.5	1.0	0.5	1.0	0.5	0.5
3	1.0	1.5	1.0	1.5	1.0	1.0

Induction of callus

After *Achyranthes bidentata* seedlings grown on MS medium for 7d, leaves, petioles and stems of seedlings were separately cut into 0.5×0.5cm^2 or 0.5-1.0cm fragments, and then were respectively cultured on 18 kinds of culture media supplemented with plant growth substances at 26±1℃ under 14h photoperiod of 1000-1200lux illumination intensity in order to study initiation and formation of callus. After explants of *Achyranthes*

bidentata seedlings had been cultured for 25d, the induction rate and the quantity of callus were counted. Furthermore, 100 explants were respectively cultured on each culture medium which was repeated three times in this study.

Statistics and analysis of data

The analysis of variance (ANOVA) and multiple comparisons of least significant difference (LSD) on the rate of callus induction in *Achyranthes bidentata* were performed with SPSS software.

Results

Callus induction from leaves of Achyranthes bidentata seedling

When cultured for 6d, the edge of most leaves curled up on, after 7d of culture, calli were found in some leaves on culture medium 2, then gradually formed on other culture media, while initiation of callus was extremely later on culture medium 17 and 18. Calli gradually enlarged along with increase of culture time, some parameters about induction and formation of callus were listed in Table 2 as cultured for 25d, in which the percentage of leaves with callus reached 100% on culture medium 1-4, 6, 8, 10, 13, 15 and 16, but was the lowest (66.7%) on culture medium 12 and 18. On the side, the mass of callus from leaves was very large on culture medium 4, 10, 13 and 14, and was big on culture medium 2, 6, 9, 17 and 18, but was small on other media.

Table 2: Callus induction from explants of *Achyranthes bidentata* on different culture medium

Culture medium	Initiation time of callus induction (d)			Rate of callus induction (%)			Callus mass		
	Leaves	Petioles	Stems	Leaves	Petioles	Stems	Leaves	Petioles	Stems
1	9	11	7	100	100	100	#	#	#
2	7	10	6	100	100	100	##	##	##
3	10	10	8	100	96.7	66.7	#	#	#
4	9	9	7	100	100	100	###	###	###
5	9	8	5	86.7	96.7	100	#	#	#
6	11	11	6	100	80	83.3	##	##	##
7	13	9	6	90	100	100	#	###	###
8	10	9	5	100	66.7	100	#	#	#
9	8	9	8	76.7	100	80	##	##	##
10	11	8	5	100	100	100	###	#	###
11	13	7	5	83.3	86.6	100	#	#	#
12	12	12	5	66.7	70	100	#	#	#
13	11	8	6	100	100	100	###	###	###
14	11	8	5	93.3	80	100	###	#	###
15	14	9	6	100	93.3	100	#	#	#
16	13	7	5	100	100	100	#	###	###
17	15	8	10	73.3	83.3	70.3	##	##	##
18	15	8	9	66.7	100	97.1	##	##	##

Note: #, ##, ### respectively indicate small, big and very large callus mass.

As shown in Table 3, effects of 6-BA and 2,4-D on callus induction from leaves were maximum, and took on extremely significant differences ($P<0.01$), KT also

significantly influenced callus induction ($P<0.05$), yet effects of other plant growth substances failed to reach statistical significance, indicating roles of plant growth substances in callus induction from leaves were obviously diverse. In addition, high concentration of 6-BA could decrease callus induction from leaves, the highest rate of callus induction (96.67%) was obtained at 0.5mg/L 6-BA, but there was no difference between rate of callus induction at 0.5mg/L 6-BA and 0mg/L 6-BA. To the contrary, high concentration of KT could promote callus induction from leaves, and the rate of callus induction was the highest with 99.86% at 1.0mg/L KT. However, 2,4-D clearly suppressed callus induction from leaves in the scope of 0.5-1.0mg/L. Extraordinary, the differences in rate of callus induction produced between various concentration of NAA, IBA or ZT were not statistically significant. Therefore, the combination of 0.5mg/L 2,4-D, 0.5mg/L 6-BA and 1.0mg/L KT might be optimum for callus induction from leaves of *Achyranthes bidentata* seedlings.

Callus induction from petioles of Achyranthes bidentata seedling

After cultured for 7d, calli formed in petioles on culture medium 11 and 16, and also were observed in most petioles when cultured for 9d, however initiation of callus was later on culture medium 1, 6 and 12. Callus existed mainly at the cut of petioles, gradually enlarged along with increase of culture time and the growth of callus got at the top as cultured for 25d. In Table 2, the percentage of petioles with callus was 100% on culture medium 1, 2, 4, 7, 9, 10, 13, 16 and 18, but was the lowest on culture medium 8, calli were observed in over 80% petioles on other culture media. Besides, the mass of callus from petioles was very large on culture medium 4, 7, 13 and 16, yet was small on other culture media except for culture medium 2, 6, 9, 17 and 18.

There was significant differences in effects of 2,4-D, IBA and NAA on callus induction from petioles (Table 4), especially effects of 2,4-D and IBA reached extremely significant differences ($P<0.01$), yet effects of 6-BA, KT and ZT were not statistically significant. Furthermore, callus induction from petioles was inhibited at the high concentration of 2,4-D, the rate of callus induction was 100% at 0.5mg/L 2,4-D, and the differences were statistically significant in rate of callus induction between 0.5mg/L and 1.0mg/L. Along with the concentration of NAA increasing, the rate of callus induction could firstly decrease and then increase, and was the highest at 1.5mg/L NNA which failed to significantly differ from that at 0.5mg/L NAA. It's probably worth noting that IBA promoted callus induction in the scope of 0.1-1.0mg/L. In addition, effects of ZT, 6-BA and KT were less and failed to reach statistical significance between various concentrations, but 6-BA and KT inhibited callus induction from petioles, especially the inhibition action of KT was stronger. Thus, the optimum combination of plant growth substances for callus induction from petioles of *Achyranthes bidentata* seedlings were 0.5mg/L 2,4-D, 0.5mg/L IBA and 0.5mg/L NAA.

Callus induction from stems of Achyranthes bidentata seedling

When cultured for 5d, calli formed at the cut of stems on culture medium 5, 8, 10-12, 14 and16, then were also found on other culture media, while initiation of callus was extremely later on culture medium 17 and 18. Along with increase of culture time, callus gradually enlarged, and

some parameters about induction and formation of callus were listed in Table 2 after 25d of culture. The percentage of stems with callus reached 100% on culture medium 1, 2, 4, 5, 7, 8, and 10-16, but was lower on culture medium 3 and 17. Besides, the mass of callus from stems was very large on culture medium 4, 7, 10, 13, 14 and 16, however was small on culture medium 1, 3, 5, 8, 11, 12 and 15.

Table 3: Effects of plant growth substance on rate of callus induction from leaves of *Achyranthes bidentata*

Plant growth substance	Average rate of callus induction (%)		
	T1	T2	T3
6-BA	91.67±1.10^{abAB}	96.67±1.51^{aA}	84.45±0.53^{Bb}
2,4-D	98.33±1.05^{aA}	89.45±0.71^{bB}	85.00±0.93^{bB}
KT	87.46±0.45^{b}	85.47±1.01^{b}	99.86±1.41^{a}
NAA	87.93±1.21	95.07±1.54	89.78±1.20
IBA	91.52±1.39	90.00±1.17	91.27±1.25
ZT	89.30±1.51	93.34±1.33	90.15±1.46

T1, T2 and T3 indicate the average rate of callus induction at level 1, level 2 and level 3 concentration of plant growth substance respectively. The different lower case and capital letter in a line separately stand for significant difference (P<0.05) or extremely significant difference (P<0.01).

Table 4: Effects of plant growth substance on rate of callus induction from petioles of *Achyranthes bidentata*

Plant growth substance	Average rate of callus induction (%)		
	T1	T2	T3
6-BA	92.22±1.31	91.67±1.11	91.67±1.21
2,4-D	100.00±1.59^{aA}	85.47±0.60^{bB}	90.08±1.05^{abAB}
KT	95.08±1.44	92.70±1.17	87.77±0.30
NAA	90.63±1.22^{ab}	88.41±1.10^{b}	96.51±1.49^{a}
IBA	83.36±1.03^{bB}	95.42±1.57^{aA}	96.77±1.50^{aA}
ZT	89.90±1.12	94.01±1.38	91.64±1.60

T1, T2 and T3 indicate the average rate of callus induction at level 1, level 2 and level 3 concentration of plant growth substance respectively. The different lower case and capital letter in a line separately stand for significant difference (P<0.05) or extremely significant difference (P<0.01).

Table 5: Effects of plant growth substance on rate of callus induction from stems of *Achyranthes bidentata*

Plant growth substance	Average rate of callus induction (%)		
	T1	T2	T3
6-BA	94.45±1.46	97.22±1.58	91.72±1.22
2,4-D	100.00±1.59^{aA}	100.00±1.47^{aA}	81.90±1.00^{bB}
KT	86.22±1.17^{b}	97.99±1.60^{a}	99.17±1.48^{a}
NAA	100.00±1.74^{aA}	90.23±1.54^{abAB}	84.21±1.32^{bB}
IBA	87.18±1.12^{b}	100.00±1.57^{a}	92.29±1.51^{ab}
ZT	98.82±1.72^{a}	85.03±1.04^{b}	99.53±1.58^{a}

T1, T2 and T3 indicate the average rate of callus induction at level 1, level 2 and level 3 concentration of plant growth substance respectively. The different lower case and capital letter in a line separately stand for significant difference (P<0.05) or extremely significant difference (P<0.01).

As described in Table 5, there were significant differences between effects produced by various concentrations of plant growth substances except for 6-BA,

particularly 2,4-D and NAA very significantly influenced callus induction from stems ($P<0.01$), but the high concentration of 2,4-D and NAA obviously restrained callus induction, and the rate of callus induction was 100% at 0.5mg/L 2,4-D, 1.0mg/L 2,4-D or 0.5mg/L NAA, respectively. In contrast, induction of callus was significantly promoted by KT and IBA, but the differences in rate of callus induction was not significant respectively between 0.5mg/L KT and 1.0mg/L KT, 1.0mg/L IBA and 0.1mg/L or 0.5mg/L IBA. Consequently, combination of 0.5mg/L 2,4-D, 0.5mg/L NAA, 0.5mg/L KT, 0.5mg/L IBA and 0.1mg/L ZT was evidently beneficial to callus induction from stems of *Achyranthes bidentata* seedlings.

Discussion

The highly differentiated cells in plant tissue could be dedifferentiated by artificial culture, and then recover to embryonic stage of cell (Skoog and Miller, 1957; Emek and Erdag, 2007; Sivakumar *et al*., 2010). In this study, callus could be induced from leaves, petioles and stems of *Achyranthes bidentata*, but it was tremendously different in initiation time, proliferation and induction rate of callus. The initiation of callus from stems occurred approximately after culture for 6d, but was later from petioles and leaves. Furthermore, compared with callus from leaves and petioles, the higher induction rate and more volume of callus from stems were observed, which was also found that the potential to form callus from leaves, stems and bulbils of *Rhizome dioscorea* was distinct (Li *et al*., 2000). Explants in different part of plant should be able to differentiate and develop on the basis of totipotency theory, but appear diverse dedifferentiation potential because cell structure, physiological status and hormone level in all kinds of organs are different (Chaturvedi *et al*., 2001; Chen *et al*., 2008; Cui *et al*., 2010). Thus, explants should be selected in order to establish system of plant regeneration and genetic transformation.

As is well known, plant growth substance plays a role in plant tissue culture, especially auxin and cytokinin could interact in many developmental processes of plant, and together alter the morphogenesis of plant cell (Nordström *et al*., 2004; Aloni *et al*., 2006; Randy *et al*., 2009). In this study, the rate of callus induction from leaves, petioles and stems of *Achyranthes bidentata* depended largely on 2,4-D, was the highest at 0.5mg/L 2,4-D, but was suppressed in the scope of 0.5-1.5mg/L 2,4-D. As also indicated in other studies, 2,4-D is important to callus induction from various explants of different plants (Dong *et al*., 2002; Sivakumar *et al*., 2010, Gao *et al*., 2011), however is not the necessary condition and easily make callus brown (Li *et al*., 2005). Furthermore, other plant growth substances were also very beneficial to callus induction from leaves, petioles and stems of *Achyranthes bidentata*, whereas their effects were various to different explants. NAA only significantly influenced callus induction from petioles and stems, but the appropriate concentration was different to petioles and stems. Contrary to NAA, effects of 6-BA were extremely significant on callus induction from leaves, however were not significant on callus induction from petioles and stems. On the other hand, KT very significantly promoted callus induction from leaves and stems and inhibited callus induction from petioles, yet IBA extremely significantly affected callus induction from petioles. In addition, ZT only significantly influenced callus induction from stems. Similarly, the

diverse responses of explants to kinds and concentrations of plant growth substances were also found in other studies (Howell *et al*., 2003; Gao *et al*., 2011). Therefore, the response to plant growth substance and the callus induction of explants were obviously different, which may be connected to diversity in physiological status, composition and metabolism of endogenous hormone between various explants of *Achyranthes bidentata*.

References

Aloni R, Aloni E, Langhans M, Ullrich C. 2006. Role of cytokinin and auxin in shaping root architecture: regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. Annals Bot. 97: 883-893.

Chaturvedi HC, Singh SK, Sharma AK, Agnihotri S. 2001. Citrus tissue culture employing vegetative explants. Indian J Exp Biol. 11: 1080-1095.

Chen MB, Zhao LY, Shang FD. 2008. Tissue culture and plantlet regeneration of *Physalis alkekengi* L. Henan Univ (Nat Sci Edi) . 38: 613-617.

Cui X, Ge C, Wang R, Wang H, Chen W, Fu Z, Jiang X, Li J, Wang Y. 2010. The BUD2 mutation affects plant architecture through altering cytokinin and auxin responses in *Arabidopsis*. Cell Res. 5: 576-586.

Dong CM, Zhang LP, Liu J. 2002. Study on tissue culture of *Achyranthes Bidentata*. Traditional Chinese Medicine. 4: 63-64.

Dragicevic V, Spasic M, Simic M, Dumanovic Z, Nikolic B. 2013. Stimulative influence of germination and growth of maize seedlings originating from aged seeds by 2,4-D potencies. Homeopathy. 102(3): 179-186.

Duan HY, Ding XS, Song JY, He YL, Zhou YQ. 2013. Plant regeneration and *Agrobacterium*-mediated transformation of *Achyranthes bidentata* using Cotton EREBP Gene. Braz Arch Biol Techn. 56(3): 349-356.

Emek Y, Erdag B. 2007. Somatic embryogenesis from leaf explants of *Gladiolus anatolicus* (Boiss.) Stapf. Pak J Biol Sci. 8: 1190-1194.

Gao J, Li J, Luo C, Yin L, Li S, Yang G, He G. 2011. Callus induction and plant regeneration in *Alternanthera philoxeroides*. Mol Biol Rep. 2: 1413-1417.

Gerszberg A, Hnatuszko-konka K, Kowalczyk T. 2015. In vitro regeneration of eight cultivars of *Brassica oleracea* var. capitata. In Vitro Cell Dev Biol Plant. 51(1): 80-87.

Howell SH, Lall S, Che P. 2003. Cytokinins and shoot development. Trends Plant Sci. 8: 453-459.

Li MJ, Li P, Hong SR. 2005. The study on callus induction and redifferentiation of *Achyranthes Bidentata* BL. Henan Normal Univ (Nat Sci Edi). 4: 118-112.

Li MJ, Xue JP, Chen MX, Guo MY, Qiu WH, Zhang JB. 2000. The influence of different factors on callus induction of *Dioscorea opposita*. Guihaia. 2: 156-160.

López BJ, Millán GM, Méndez BA, Morquecho CA, Ramírez CE, Molina TJ. 2007. Cytokinin receptors are involved in alkamide regulation of root and shoot development in *Arabidopsis*. Plant Physiol. 145: 1703-1713.

Melissa SP, Edward LH, Adrian DH, Michael RS. 2006. A transcriptome based characterization of habituation in plant tissue culture. Plant Physiol. 140: 1255-1278.

Nordström A, Tarkowski P, Tarkowska D, Norbaek R, Astot C, Dolezal K, Sandberg G. 2004. Auxin regulation of cytokinin biosynthesis in *Arabidopsis*

thaliana: a factor of potential importance for auxin-cytokinin-regulated development. Proc Natl Acad Sci USA. 101: 8039-8044.

Randy OC, Hexon CC, Lourdes MR, Jose LB. 2009. The role of microbial signals in plant growth and development. Plant Signal Behav. 8: 701-712.

Sivakumar P, Law YS, Ho CL, Harikrishna JA. 2010. High frequency plant regeneration from mature seed of elite, recalcitrant Malaysian indica rice (*Oryza sativa* L.) CV. MR 219. Acta Biol Hung. 3: 313-321.

Skoog F, Miller CO. 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. In: Biological Action of Growth Substances. Symp Soc Expt Biol. 11: 118-131.

Zhao XM, Xu GZ, Li JL. 2004. Progress in modern pharmacological study of *radix cyathulae* and *Achyranthes bidentata*. West China J Pharm Sci. 3: 205-207.