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Effects of plant growth substances on callus re-differentiation of medicinal plant *Achyranthes bidentata*

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Abstract

In this study, callus from stem could easily differentiate to form bud and root, while re-differentiation potential of callus from leaf and petiole was lower, especially bud was not discovered in callus from petiole. Compared with other plant growth substances, effect of 2,4-D on callus re-differentiation of various explants was extremely significant, simultaneously the interaction among plant growth substances was also discovered, in which the optimum combination of plant growth substances for bud and root formation from callus of stem was respectively as follows, combination of 0.5mg/L 2,4-D, 1.0mg/L NAA, 0.1mg/L IBA and 0.1mg/L ZT, and combination of 0.5mg/L 2,4-D, 1.0mg/L KT, 0.5mg/L NAA and 0.5mg/L IBA. Besides, combination of plant growth substances for root formation from callus of leaf and petiole was also obtained. In brief, the re-differentiation capacity of callus from various explants was different, and roles of plant growth substances in re-differentiation were also significantly diverse, thus these results would provide theory basis for tissue culture and plant regeneration of *Achyranthes bidentata*.

Keywords: *Achyranthes bidentata*, plant growth substance, callus, bud, root.
Abbreviations: NAA_naphthylacetic acid; 6-BA_6-benzylaminopurine; KT_kinetin; IBA_indale butyric acid; 2,4-D_2, 4-dichlorophenoxyacetic acid; ZT_zeatin.

Introduction

Achyranthes bidentata belongs to *Achyranthes* L. of Amaranthaceae, and is frequently used in cure health because it has many medicinal effects such as nourishing liver and kidney, strengthening sinews and bones, falling blood pressure, invigorating blood circulation silt, analgesia, and so on (Li, 1986; Xu et al., 2002; Zhao et al., 2004). Recently, study on herbalism, cultivation technique, active ingredient and pharmacologic effect of *Achyranthes bidentata* has rapidly progressed, tissue culture of *Achyranthes bidentata* has also been reported, but regeneration frequency of explants is low, and even appeared obviously diverse to same explant in different research (Dong et al., 2002; Li et al., 2004). As is well known, plant growth substance is very important to induction and differentiation of callus (Nordstrom et al., 2004; Aloni et al., 2006; Jose et al., 2007), especially auxin and cytokinin could collectively alter morphogenesis of plant cell and induce plant cell to form particular tissues such as callus, shoot, root, or a whole plant (Melissa et al., 2006; Cui et al., 2010). At present, NAA, 2,4-D, 6-BA, KT and other plant growth substances have been combinedly used in plant tissue culture (Fan, 2003; Nordstrom et al., 2004; Aloni et al., 2006; Randy et al., 2009), therefore kind and concentration of plant growth substance need to be selected in plant tissue culture. Owing to saving time and effort, orthogonal design is fully effective to option of optimum experiment program and has been successfully applied in plant tissue culture (Du and Wang, 1999; Li et al., 2001; Xuan et al., 2004; Chen et al., 2007).

In this study, effects of 6-BA, 2,4-D, KT, NAA, IBA and ZT on callus re-differentiation of *Achyranthes bidentata* were explored by orthogonal design in order to establish efficient system of plant regeneration and provide theory basis for plant tissue culture and genetics breeding of *Achyranthes bidentata*.

Results

Bud differentiation from callus of Achyranthes bidentata

When cultured for 13d, the green bud points were found at the edge of calli from stem on culture medium 15, and then cluster buds were formed, later could be discovered in calli from stem on culture medium 1 and 4, however bud differentiation on other culture media was initiated later. As cultured for 30d, bud differentiation of callus from different explants displayed prodigious diversity, bud could be formed in most calli from stem, while was merely formed in 0.3% calli from leaf, and could not be formed in calli from petiole. On different culture medium, bud formation in callus from stem was shown in Table 2, the frequency of calli with bud reached 33.3% on culture medium 1, 4, 13 and 16, was 22.2% on culture medium 7 and 15, and lower on culture medium 2, 8-12 and 17, however bud failed to be formed on other media which was not listed in Table 2. Furthermore, there are differences between effects of plant growth substances on bud formation in callus from stem (Table 3), 2,4-D, NAA and IBA significantly influenced bud formation, especially effects of 2,4-D reached extremely significant differences ($P<0.01$), yet effects of 6-BA, ZT and KT were not statistically significant. The differences between effects produced by various concentration of plant growth substance were also found. The rate of bud formation can significantly decrease firstly and then increase in response

to increase of 2,4-D and IBA, in which low or high concentration of NAA suppressed bud formation, and bud formation was obviously restrained by KT. Thus, combination of 0.5mg/L 2,4-D, 1.0mg/L NAA, 0.1mg/L IBA and 0.1mg/L ZT was evidently beneficial to bud formation from callus of stem.

Root formation in callus from leaf of Achyranthes bidentata

After 9d of culture, hairy roots were formed in calli on culture medium 1, and then on culture medium 2 and 4. At 17d, hairy roots were formed on culture medium 3, as cultured for 25d or so, hairy roots were also found on culture medium 5, 8-12, 14 and 15, but differentiation of callus was later on culture medium 13, 16 and 17. When cultured for 40d, the statistic results about root formation were demonstrated in Table 4, the frequency of calli with root on culture medium 1-4 and 11 was 100%, was 83.3% on culture medium 10, however the potential of callus to form root on other culture media was lower, especially only 16.7% on culture medium 8 and 17, moreover root was not formed on culture medium 6, 7 and 18. As described in Table 5, effects of 6-BA on root formation reached extremely significant differences ($P<0.01$), 2,4-D, KT and NAA also significantly influenced root formation ($P<0.05$), yet effects of IBA and ZT were less and did not reach statistical significance. Furthermore, root formation was significantly restrained by 6-BA and KT, particularly obvious in 6-BA, rate of root formation also decreased in response to high or low concentration of NAA, and the highest was reached at 1.0mg/L NAA. Besides, along with increase in concentration of 2,4-D, rate of root formation could firstly decrease and then increase, and there was significant difference between effects of 0.5mg/L 2,4-D and 1.5mg/L 2,4-D. Therefore, combination of 1.5mg/L 2,4-D, 1.0mg/L NAA and 0.1mg/L ZT might be optimum for root formation from callus of leaf.

Root formation in callus from petiole of Achyranthes bidentata

The hairy roots were observed in calli from petiole on culture medium 1 and 16 when cultured for 19d, then on culture medium 2, however root formation was later on other culture media. As shown in Table 4, the frequency of callus with root on culture medium 1 and 2 was 66.7%, was lower (16.7%) on culture medium 4, 5, 10, 12, 13, 15 and 16, nevertheless root was not formed on other media. There were differences in effects of plant growth substances on root formation (Table 6), 6-BA, 2,4-D and ZT had very significant influence on root formation, especially effects of 6-BA reached extremely significant differences ($P<0.01$), yet effects of other plant growth substances were not statistically significant. In Table 6, rate of root formation descended along with increase in concentration of 6-BA, 2,4-D and ZT, particularly was very evident in 6-BA, furthermore, the differences between various concentration of 6-BA, 0.5-1.0 mg/L 2,4-D and 1.5 mg/L 2,4-D, or 0.1 mg/L ZT and 1.0 mg/L ZT reached statistically significance. However, effects of KT, NAA and IBA failed to reach statistical significance between various concentration. Therefore, the optimum combination of plant growth substance for root formation from callus of petiole was 0.5mg/L 2,4-D and 0.1mg/L ZT.

Root formation in callus from stem of Achyranthes bidentata

When calli from stem were cultured for 10d, hairy root was formed on culture medium 8, 10 and 12, soon afterwards formed on culture medium 1, 4 and 14, but root was not formed on culture medium 6, 9, 11, 13, 16 and18 after cultured for 30d. As shown in Table 4, hairy roots were formed on 18 kinds of culture media, especially the frequency of calli with root was 100% on culture medium 1-3 and 10, however was lower on other media, and merely 16.7% on culture medium 4, 7, 8 and 16-18. As indictated in Table 7, there were significant differences between effects of plant growth substances except for ZT, particularly 6-BA very significantly restrained on root formation ($P<0.01$), root formation was also obviously restrained by higher concentration of 2,4-D, but KT evidently promoted root formation. Besides, along with increase in concentration of NAA, rate of root formation can firstly decrease and then increase, and differences between effects of 1.0mg/L NAA and 0.5mg/L NAA or 1.5mg/L NAA were statistically significant. High or low concentration of IBA failed to significantly influence root formation,and there was no significant difference between effects produced by various concentration of ZT. Thus, combination of 0.5mg/L 2,4-D, 1.0mg/L KT, 0.5mg/L NAA and 0.5mg/L IBA was evidently beneficial to root formation from callus of stem.

Discussion

The highly differentiated cell in plant tissue could be dedifferentiated by artifical culture, and then recover to embryonic stage of cell (Skoog and Miller, 1957; Murashige and Skoog, 1962; Evans and Sharp, 1981; Emek and Erdag, 2007; Sivakumar et al., 2010). It is well known, re-differentiation of callus is influenced by many factors in plant tissue culture, and major factor is plant growth substance which has evident effects and is critical to growth and differentiation of plant cell and tissue, induction and formation of bud and root (Himanen et al., 2002; Fan, 2003; Melissa et al., 2006; Claudia-Anahí et al., 2008; Randy et al., 2009). In this study, functions of 2,4-D, NAA and IBA were significant in bud differentiation of callus from stems, especially 2,4-D extremely influenced bud differentiation, and bud formation in callus from stem was absolutely influenced by interaction among plant growth substances, which was also found in many studies (Nordstrom et al., 2004; Aloni et al., 2006; Jose et al., 2007). On the other hand, effects of 2,4-D on root formation from callus of leaf, petiole and stem were also significant, but the potential to form root in callus from various explants was different in the scope of 0.5-1.5mg/L 2,4-D. Also, effects of NAA on root formation in callus from leaf and stem were significant, however 6-BA significantly inhibited root formation in callus from leaf, petiole and stem, furthermore root formation in callus from leaf was also evidently inhibited by KT which obviously promoted root formation in callus from stem. IBA only influenced significantly root formation in callus from stem, while effects of ZT on root formation were not significant. Besides, this research also stated clearly that root fromation from callus was evidently influenced by the synergistic, antagonistic or additive interactions among plant growth substances, which was consistent with results in other studies (Nordstrom et al., 2004; Aloni et al., 2006; Jose et al., 2007). In addition, callus re-differentiation of different explants from *Achyranthes bidentata* displayed prodigious diversity, callus from stem could easily differentiate to form bud and root, root and bud formation rate of callus from leaf was lower, and the re-differentiation potential of callus from petiole was relatively low, roots were only found on approximately 30% calli and the bud was not found, while petiole of *Taxus chinensis* had strong re-differentiation potential as compared with other explants (Ye, 2008). Generally, explants in different part of plant should be potential to re-differentiate and develop according to totipotency theory, but appear diverse

Table 1. Factors and levels 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

Table 2. Bud formation in callus from stem on different culture medium

Culture medium / Index	1	2	4	7	8	9	10	11	12	13	15	16	17
Day of bud initiation (d)	17	27	15	26	25	25	29	27	29	26	13	27	30
Rate of bud formation (%)	33.3	11.1	33.3	22.2	11.1	11.1	11.1	11.1	11.1	33.3	22.2	33.3	11.1

Note: the culture medium was respectively supplemented with various concentrations of 6-BA, 2,4-D, KT, NAA, IBA or ZT according to Table 1, so concentration and kind of plant growth substance may be different in every culture medium.

Table 3. Effects of plant growth substance on bud formation in callus from stem

Index / Factor	Average rate of bud formation (%)		
	T1	T2	T3
6-BA	12.950±0.225	14.817±0.420	14.800±0.376
2,4-D	27.750±0.559 A	0.445±0.024 C	15.261 ±0.319 B
KT	19.237±0.514	15.864±0.393	7.466±0.312
NAA	5.785±0.128 b	25.120± 0.435 a	11.662±0.325 b
IBA	20.435±0.316 a	5.354±0.115 b	16.779±0.213 a
ZT	12.838±0.319	18.713±0.507	11.015±0.314

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

re-differentiation potential because of different cell structure and physiological status, and levels of endogenous hormones in all kinds of organs also are various (Skoog and Miller, 1957; Chen et al., 2004; Chen et al., 2008; Cui et al., 2010). Furthermore, responses of callus from different explants of *Achyranthes bidentata* to kind and concentration of plant growth substance were also diverse. Therfore, it was supposed that re-differentiation of callus is probably related to composition, transportation, secretion and distribution of more hormone in organ and tissue 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 soaked for 12h at 24℃, seeds were surface-sterilized for 30s with 75% ethanol, subsequently deep-sterilized for 6min with 0.1% mercury bichloride, and then washed five times with sterile water. The aseptic seeds were cultured on MS culture medium at 26±1℃ with 14h photoperiod of 1000-1200lux illumination intensity, when cultured for 7d, leaves, petioles and stems of seedlings were used as explants, separately cut into 0.5×0.5cm^2 or 0.5-1.0cm fragments.

Experimental design

In this study, MS culture medium was used as minimal medium, various concentrations of 6-BA, 2,4-D, KT, NAA, IBA or ZT were respectively added into MS culture medium to explore their effects on callus re-differentiation of *Achyranthes bidentata*. The orthogonal design of L_{18} (3^7) (Li et al., 1997) was adopted, factors and its concentration were described in Table 1. 18 kinds of culture media supplemented with plant growth substances were designed according to orthogonal array, each culture medium was composed of 3.0% sugar, 0.7% agar and different concenration of plant growth substances, and pH of culture medium was approximately 7.0.

Re-differentiation of callus

Callus cultures were initiated from leaves, petioles and stems of *Achyranthes bidentata* on MS culture medium supplemented with 0.5mg/L 2,4-D and 0.5mg/L NAA at 26±1℃ under 14h photoperiod of 1000-1200lux illumination intensity. After 30d, callus from various explants was cut into small pieces and then subcultured on 18 kinds of culture media with different kind and concentration of plant growth substances at 26±1℃ under 14h photoperiod of 1000-1200lux illumination intensity to study callus re-differentiation and formation of bud and root. Besides, in this experiment, one hundred calli were cultured on each culture medium which was repeated three times.

Statistics and analysis of data

Range (R), analysis of variance (ANOVA) and multiple comparisons of least significant difference (LSD) on rate of bud and root formation in callus from *Achyranthes bidentata* were performed by SPSS software.

Table 4. Root formation in callus from *Achyranthes bidentata* on different culture medium

Index / Culture medium	Day of root initiation (d)			Rate of root formation (%)		
	Leaf	Petiole	Stem	Leaf	Petiole	Stem
1	9	19	15	100%	66.7%	100%
2	12	23	21	100%	66.7%	100%
3	17	0	22	100%	0	100%
4	14	30	12	100%	16.7%	16.7%
5	25	28	25	33.3%	16.7%	50%
6	0	0	32	0	0	33.3%
7	0	0	27	0	0	16.7%
8	26	0	10	16.7%	0	16.7%
9	24	0	34	66.7%	0	33.3%
10	24	28	10	83.3%	16.7%	100%
11	24	0	34	100%	0	33.3%
12	25	31	10	66.7%	16.7%	33.3%
13	30	28	34	33.3%	16.7%	66.7%
14	25	0	14	33.3%	0	50%
15	25	28	24	66.7%	16.7%	50%
16	30	19	33	33.3%	16.7%	16.7%
17	30	0	28	16.7%	0	16.7%
18	0	0	31	0	0	16.7%

Table 5. Effects of plant growth substance on root formation in callus from leaf

Index / Factor	Average rate of root formation (%)		
	T1	T2	T3
6-BA	91.667±1.501 A	44.433±0.809 B	22.233±0.471 C
2,4-D	58.317±1.135 ab	28.440±0.613 b	71.576±1.397 a
KT	74.845±1.453 a	46.548±0.931 b	36.939±0.717 b
NAA	19.096±0.503 b	78.971±1.379 a	60.266±1.039 ab
IBA	53.595±0.949	42.870±0.927	61.868±1.216
ZT	49.232±0.998	65.463±1.319	43.638±0.995

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

Table 6. Effects of plant growth substance on root formation in callus from petiole

Index / Factor	Average rate of root formation (%)		
	T1	T2	T3
6-BA	27.800±0.631 A	11.133±0.315 B	2.783±0.089 C
2,4-D	22.250±0.524 a	14.067±0.397 a	5.399±0.176 b
KT	9.684±0.213	19.302±0.509	12.731±0.308
NAA	19.379±0.522	15.591±0.419	6.747±0.213
IBA	14.449±0.418	19.426±0.620	7.842±0.109
ZT	19.974±0.689 a	13.941±0.386 ab	7.801±0.190 b

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

Table 7. Effects of plant growth substance on root formation in callus from stem

Index / Factor	Average rate of root formation (%)		
	T1	T2	T3
6-BA	77.767±1.313 A	44.450±0.856 B	19.467±0.553 C
2,4-D	52.800±0.981 a	56.875± 1.124 a	32.009±0.706 b
KT	38.204±0.854 b	41.764±0.813 b	61.716±1.307 a
NAA	54.229±1.029 a	26.931±0.705 b	60.523±1.128 a
IBA	39.307 ±0.974 b	61.845 ±1.267 a	40.531±0.831 b
ZT	51.135±1.091	43.721±0.929	46.827±0.996

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

Conclusion

Plant growth substances are very critical to growth and differentiation of cell and tissue, induction and formation of bud and root, and so on. However, there are synergistic, antagonistic or additive interactions between cytokinins and auxins, and the varying ratios of cytokinin and auxin could induce plant cells to form particular tissues. In this study, compared with callus from leaf and petiole of *Achyranthes bidentata*, callus from stem could easily differentiate to form bud and root, in which functions of 2,4-D were extremely significant, the interaction among plant growth substances was also found, and the optimum combination of plant growth substances for formation of bud and root from stem was obtained. Accordingly, these results would provide theory basis for plant tissue culture and genetics breeding of *Achyranthes bidentata*.

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