In vitro micropropagation of Nopalxochia ackermannii Kunth

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Abstract. The effects of dark culture, plant growth regulators, culture temperature and transplantation matrices on micropropagation of *Nopalxochia ackermannii* were studied. The results showed that light was necessary for the seed germination. The suitable medium for the seed germination was Murashige and Skoog medium (MS) supplemented with 0.5 mg·L^{-1} 6-benzyladenine (BA) and 0.25 mg·L^{-1} indole-3-butyric acid (IBA). Zeatin (ZT) was more suitable for the multiplication than kinetin (KT) and BA, especially in combination with a-napthalene acetic acid (NAA). The suitable medium for the multiplication was MS basal medium supplemented with 0.5 mg·L^{-1} ZT, 0.2 mg·L^{-1} NAA and 500 mg·L^{-1} casein hydrolysate (CH), on which 100.00% induction rate of shoots and 6.76 multiplication coefficient were obtained. 25℃ culture temperature was beneficial for proliferation and growth of shoots. The medium of half-strength MS with NAA 0.5 mg·L^{-1} was suitable for rooting, on which 94.21% shoots could be rooted with a root number of 5.90. After being transplanted into the mixture of pearlite and humus soil (1:1), 91.67% plantlets survived and grew well.

1.Introduction

Nopalxochia ackermannii Kunth is an important plant species in the Cactaceae family. It's one of the most widely cultured succulent plants and it is as an ornamental plant for the bizarre and particular morphological traits including bell-shaped colorful aromatic flower and green cladode. It can be cultured as a multi-purpose plant since its stems, flowers and fruits are commonly used as forages, vegetables, foods and medicines [1-2].

The common propagation methods of *N. ackermannii* were cutting and grafting, but the reproductive rates were low because of seasons, easy decay of cuttings or scions and some other factors [3-4]. Meanwhile, using tradition-al asexual propagation like cutting and grafting for a long time can spread diseases which cause variety degenera-tion [5]. Using tissue culture method can get not only high propagation rate, but also a rejuvenation effect [6-8]. Although the studies on *in vitro* culture and plant regeneration of Cactaceae family have achieved success [9-13], there are only two cases of report on culture *in vitro* of *Nopalxochia* genus. Liu *et al.* induced adventi-tious buds and regenerated plants from callus of stem segments [14]. Ni *et al.* used seeds of *N. ackermannii* as explants to induce the sterile seedlings and used stems excised from *in vitro* seedlings to proliferate [15]. But Liu et al. didn't study proliferation of shoots and transplantation of plantlets. Ni *et al.* didn't systematically study the media and culture condition of seed germination, shoot multiplication and rooting. Furthermore, using callus to induce regeneration of shoots had some problems such as poor genetic stability, long plant regeneration cycle and the declined ability of regenerated plants with the long-term subculture [16-18].

Using seeds and stem segments as explants, the effects of dark culture, plant growth regulators, culture temperature and transplantation medium on micropro-pagation of *N. ackermannii* were studied in this experiment, aiming to establish a more perfect *in vitro* regeneration system and provide theoretical and technical basis for utilization and resource conservation of *N. ackermannii* and other succulent plants.

2.Materials and methods

2.1 Plant material

The hybrid seeds of *N. ackermannii* × (*N. ackermannii*×*N. anguliger*), were used as explants to establish the sterile seedlings. The seedlings were used in subsequent experiments.

2.2 In vitro germination

The seeds were imbibed in distilled water for 24 h, then surface sterilized with 0.1% $HgC1_2$ for 15 min, rinsed in sterile distilled water and germinated aseptically on Murashige and Skoog medium (MS) supplemented with 0.25 mg·L^{-1} indole-3-butyric acid (IBA), 0.5 or 1.0 mg·L^{-1} 6-benzyladenine (BA), 3.0 g·L^{-1} activated charcoal (AC), 2% sucrose and 0.6% agar for 0 and 28 days dark culture, respectively. The germination rate and plant growth condition was tested after 28 d and 56 d culture.

2.3 Shoot formation, multiplication and rooting

Stem segments (1 cm in length) excised from *in vitro* seedlings were subcultured on MS medium containing different concentrations of BA, kinetin (KT), zeatin (ZT) and a-napthalene acetic acid (NAA). The concentrations of BA were 0.0, 0.5, 1.0, 2.0, 3.0, and 4.0 mg·L^{-1}, KT 0.0, 0.5, and 1.0 mg·L^{-1}, ZT 0.0, 0.25, 0.5, 0.75, and 1.0 mg·L^{-1}, and NAA 0.0, 0.1, and 0.2 mg·L^{-1}, respectively, resulting in 21 combinadons in total (Table 1 and Table 2). For the cultural temperature test, the stem segments inoculated on MS medium containing 1.0 mg·L^{-1} ZT and 0.1 mg·L^{-1} NAA were maintained at 20℃, 25℃, and 30℃, respectively. All the media contained casein hydrolysate (CH) 500 mg·L^{-1}, 2% sucrose and 0.6% agar, which were adjusted to pH 5.8. After 8 weeks of culture, the number and height of newly formed shoots were recorded.

Microshoots (2cm in length) were incubated on half-strength MS medium supplemented with different concentrations of NAA and CH to improve the formation of roots. The concentrations of NAA were 0.5 and 1.0 mg·L^{-1}, and those of CH were 0 and 500 mg·L^{-1}. The media were solidified with 0.6% agar and supplemented with 2% sucrose. After 7 weeks of culture, the percentage of root formation, the number and length of the formed roots were recorded.

Table 1. Effects of concentrations of BA and KT on multiplication of *N. ackermannii*.

BA (mg·L^{-1})	KT (mg·L^{-1})	NAA (mg·L^{-1})	Number of explants	Induction rate of shoots (%)	Multiplication coefficient	Performance of shoot growth
0.5	0.0	0.2	65	35.38 cd	1.56 e	Weakest and shorter
1.0	0.0	0.2	60	25.00 e	1.32 f	Weakest and shorter
2.0	0.0	0.2	60	28.33 de	1.38 ef	Weakest and shorter
3.0	0.0	0.2	63	42.86 c	1.98 d	Weakest and shorter
4.0	0.0	0.2	66	42.42 c	1.52 ef	Weakest and shorter
0.0	0.5	0.0	51	74.51 b	2.39 c	Weaker and shortest
0.0	0.5	0.2	69	89.86 a	2.80 b	Weak and short
0.0	1.0	0.0	74	67.56 b	1.96 d	Weaker and shorter
0.0	1.0	0.2	56	98.24 a	4.47 a	Sturdy and longer

The English lowercase letters after the same column of data indicate significant differences between different treatments ($p < 0.05$). Similarly hereinafter.

Table 2. Effects of concentrations of ZT and NAA on multiplication of *N. ackermannii*.

ZT (mg·L^{-1})	NAA (mg·L^{-1})	Number of explants	Induction rate of shoots (%)	Multiplication coefficient	Performance of shoot growth

0.25	0.0	191	86.85 d	3.36 gh	Sturdy and shorter
0.25	0.1	168	95.24 bc	3.80 f	Sturdy and short
0.25	0.2	192	86.92 d	3.73 fg	Rather sturdy and short
0.5	0.0	251	89.15 cd	3.79 f	Sturdy and shorter
0.5	0.1	189	99.47 ab	6.02 b	Rather sturdy and long
0.5	0.2	178	100.00 a	6.76 a	Rather sturdy and longer
0.75	0.0	196	84.15 de	3.10 h	Rather sturdy and short
0.75	0.1	363	84.26 de	3.26 h	Rather sturdy and longest
0.75	0.2	240	86.67 d	4.33 e	Rather sturdy and longer
1.0	0.0	248	72.09 e	2.70 i	Sturdy and long
1.0	0.1	272	86.37 d	5.03 d	Rather sturdy and longer
1.0	0.2	320	86.83 d	5.48 c	Rather sturdy and longer

2.4 Transplantation

Rooted plantlets (3~4 cm in length) were carefully removed from the culture vessels, washed in tap-water to eliminate excess of agar and then transplanted into four mixed media as follows: (1) pearlite and humus soil (1:1), (2) vermiculite and humus soil (1:1), (3) sand and humus soil (1:1), (4) vermiculite, pearlite and humus soil (2:1:3). After 21 days of transplantation, the plants were sprayed with Yangkang microorganism fertilizer 500 fold once every 7 d. The survival rate and length of plants were recorded after 2 months of transplantation.

2.5 Culture conditions

In addition to the special statement, cultures were incubated at 25±2℃ under 14 h photoperiod provided by white fluorescent lights.

2.6 Statistical analysis

All the experiments were repeated three times and the standard deviation and standard error were calculated using at least 17 explants per treatment. The data were subject to analysis of variance (ANOVA) to assess treatment differences and interactions using the SPSS statistical package for windows (release 11.0, SPSS INC). Significance between means was tested by Duncan's multiple range test.

3.Results

3.1. Seed germination

After being cultured 28 d, the seeds in light culture all germinated, compared with only 1.6% of the seeds in dark culture. After the seeds in dark culture were transferred to light culture, they germinated gradually after 7 d of culture. The seed germination rate increased to 75.8% after 28 d of culture. But their plant heights (1.89 cm) were obviously lower than those (2.63 cm) in light culture all along. It was thus clear that light culture was good for *N. ackermannii*'s seed germination and growth, and the seeds should be light-dependent type.

After being cultured 56 d, the germination rates of seeds on culture media containing 0.5 $mg \cdot L^{-1}$ BA, 0.25 $mg \cdot L^{-1}$ IBA were 85.7% and 87.5%, respectively. Meanwhile, the results showed that AC had no significant effect on the seed germination and growth. Therefore, MS basal medium supplemented with 0.5 $mg \cdot L^{-1}$ BA and 0.25 $mg \cdot L^{-1}$ IBA was the suitable medium for seed germination of *N. ackermannii*.

3.2 Effects of plant growth regulators on multiplication

It was indicated that there were significant differences in interactive effects of BA, KT, ZT, and NAA on the induction rate of new shoots and shoot multiplication among these media.

The induction rate and the multiplication coefficient of shoots cultured on MS medium supplemented with 0.2 mg·L^{-1} NAA and 0.5, 1.0, 2.0, 3.0, and 4.0 mg·L^{-1} BA were low, only 25.00%~42.86% and 1.32~1.98, respectively (Table 1). And the shoots grew weakly. Table 1 showed that both of induction rate and multiplication coefficient of shoots increased obviously when keeping the same NAA concentration but instead BA with KT. The explants cultured on MS medium supplemented with 1.0 mg·L^{-1} KT and 0.2 mg·L^{-1} NAA exhibited the largest induction rate and multiplication coefficient (98.24% and 4.47, respectively). Besides, using KT alone could increase the induction rate of newly formed shoots, but the multiplication coefficients of shoots were low (1.96~2.39), and the shoots grew weakly.

When using ZT in place of KT, both induction rate and multiplication coefficient of shoots increased and the shoots grew strongly (Fig. 1A and Fig. 1B). Especially, the explants cultured on MS medium supplemented with 0.5 mg·L^{-1} ZT and 0.2 mg·L^{-1} NAA exhibited the highest induction rate (100%), the largest multiplication coefficient (6.76) and the most sturdy shoots as shown in Table 2. By comparing induction rate, multiplication coefficient and growth performance of shoots, it could be concluded that the suitable medium for *N. ackermannii* multiplication was MS basal medium supplemented with 0.5 mg·L^{-1} ZT, 0.2 mg·L^{-1} NAA and 500 mg·L^{-1} CH.

3.3 Effects of culture temperature on multiplication

Shoots of *N. ackermannii* cultured on MS medium containing 1.0 mg·L^{-1} ZT, 0.1 mg·L^{-1} NAA and 500 mg·L^{-1} CH were maintained at different levels of temperature. There were very significant differences of proliferation and growth situation among the treatments (Table 3). The explants cultured at 20℃ produced the weakest and shortest shoots, with the lowest multiplication coefficient and the highest death rate. However, the explants cultured at 25℃ produced rather sturdy and long shoots, with the lowest death rate and the largest multiplication coefficient (Fig. 1B). Higher number and length of shoots were observed when explants were cultured at 30℃ (Fig. 1C), but 28.33% shoots were vitreous (Fig. 1D). Therefore, under the same conditions, 25℃ was the optimum culture temperature for proliferation and growth of *N. ackermannii*'s shoots.

Table 3. Effects of different culture temperatures on multiplication of *N. ackermannii*

Culture temperature (℃)	Number of explants	Induction rate of shoots (%)	Multiplication coefficient	Mortality rate of shoots (%)	Performance of shoot growth
20	217	46.52 b	1.80 b	23.55 a	Weak and short
25	272	86.37 a	5.03 a	1.20 c	Rather sturdy and long
30	189	87.30 a	5.01 a	10.19 b	Thin and long

3.4 Rooting

It was found that some of the shoots cultured on the proliferation media containing NAA developed a number of thin and short roots at their base. These rooted plants failed to grow in soil. Lots of roots were induced at the base of the shoots after 28 d~42 d of culturte when isolated shoots were incubated on half-strength MS medium supplemented with different concentrations of NAA and CH. 89.71%~94.21% of shoots cultured on all the rooting media produced 5.72~6.93 main roots with flourish fibrous roots after 7 weeks of culturte as shown in Table 4 (Fig. 1E). Table 4 showed that there were no significant differences in interactive effects of NAA and CH on rooting rate and root numbers between these media. There were significant effects of NAA on root length and the length of roots were lower with the increase of NAA. The highest of rooting rate and higher number and length of roots were observed when shoots were cultured on half-strength MS medium supplemented with 0.5 mg·L^{-1} NAA.

3.5 Transplantation

The plantlets were transplanted into 4 kinds of mixed matrices, as follows: pearlite and humus soil, vermiculite and humus soil, sand and humus soil, vermiculite, pearlite and humus soil. It was observed that the plantlet survival rates were 91.67%, 85.87%, 61.39%, and 72.73%, with 3.93 cm, 3.79 cm,

1.95 cm, and 2.64 cm growth increment, respectively. 50.00% of plantlets transplanted into the mixed matrix of pearlite and humus soil sprouted new shoots after 2 months of transplantation. The upper part of the stems of the plants turned cylindrical into flat after 4 months of transplantation (Fig. 1F).

Table 4. Effect of combination of NAA and CH on rooting of *N. ackermannii*.

NAA ($mg \cdot L^{-1}$)	CH ($mg \cdot L^{-1}$)	Number of explants	Rooting rate (%)	Number of roots	Length of roots (cm)	Induction rate of shoots (%)
0.5	0.0	138	94.21 a	5.90 a	2.41 a	85.52 ab
0.5	0.5	136	89.71 a	5.72 a	2.73 a	86.03 ab
1.0	0.0	132	93.94 a	6.93 a	1.44 b	77.27 b
1.0	0.5	138	93.50 a	6.24 a	1.73 b	91.30 a

Figure 1. Micropropagation of *N. ackermannii* Kunth A The explants cultured on MS medium supplemented with ZT. B The explants cultured at 25℃on MS medium supplemented with KT. C Higher number and length of shoots were observed when explants were cultured at 30℃. D The explants were vitrified at 30 ℃. E The growth of root on half-strength MS medium supplemented with NAA and CH. F The upper part of the stems of the plants turned cylindrical into flat after 4 months of transplantation.

4.Discussion

Ni gained the sterile seedlings from seeds of *N. ackermannii* after culturing 2 generations (at least 60 d), then used the stems excised from the in vitro seedlings to proliferate when the stems were 1cm high [15]. Liu regenerated plants of *N. ackermannii* from calluses of stem segments [14]. Both of their culture programs were complicate with long period and low multiplication efficiency. By studying with the light condition and hormone combination in seed germination *in vitro*, medium formula and culture temperature in proliferation of shoots, the present experiment showed that the seeds of *N. ackermannii* were light-dependent type, strong seedlings were gained from seeds, and sturdy shoots with high multiplication coefficient were successfully induced from stem segments.

In previous reports about *in vitro* culture, BA, KT, and ZT were used for shoot induction and proliferation in many plants, but they are seldom used together *in vitro* culture of cactus plants [19-

27]. Studies were carried out of the effects of BA, KT and ZT on shoot proliferation of *N. ackermannii* in the present experiment. The results showed that BA had no significant promotion on proliferation and growth of shoots of *N. ackermannii*. KT could promote the sprouting of stem axillary buds of *N. ackermannii*, but most shoots grew weakly. The addition of ZT to the media, especially in the combination of ZT and NAA, could make *N. ackermannii*'s axillary buds sprout earlier, gain high multiplication coefficient, and get strong shoots.

Compared with the previous researches in which IBA or IBA was used in combination with NAA and activated charcoal to induce rooting of *N. ackermannii*'s shoots, only NAA was used, which was low in cost, but got strong plantlets with high rooting rate and induction rate of new shoots in the present experiment.

It was also found that lots of shoots suffered from vitrification when explants were cultured on semisolid multiplication media at 25℃ and solid multiplication media at 30℃. This might be due to *N. ackermannii* being typically Cactaceous succulent plant and prone to serious vitrification on semisolid media at high temperature causing high relative humidity in the culture vessels. Therefore, semisolid culture should not be adopted for *in vitro* culture of Cactaceous plants, and culture vessels with good air permeability should be adopted for sealing.

5.Conclusion

The results showed that light was necessary for the seed germination. The suitable medium for the seed germination was MS supplemented with 0.5 $mg \cdot L^{-1}$ BA and 0.25 $mg \cdot L^{-1}$ IBA. ZT was more suitable for the multiplication than KT and BA, especially in combination with NAA. The suitable medium for the multiplication was MS basal medium supplemented with 0.5 $mg \cdot L^{-1}$ ZT, 0.2 $mg \cdot L^{-1}$ NAA, and 500 $mg \cdot L^{-1}$ CH, on which 100.00% induction rate of shoots and 6.76 multiplication coefficient were obtained. 25℃ culture temperature was beneficial for proliferation and growth of shoots. The medium of half-strength MS with NAA 0.5 $mg \cdot L^{-1}$ was suitable for rooting, on which 94.21% shoots could be rooted with a root number of 5.90. After being transplanted into the mixture of pearlite and humus soil (1:1), 91.67% plantlets survived and grew well.

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