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Full Length Article

Establishment of Stem Node Regeneration System for Maize (*Zea mays*)

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Abstract

As explants, maize stem nodes reduce a series of complex processes such as induction and differentiation compared with other receptor materials. In this study, four maize inbred lines (GSH9901, H4065, H4077 and H4051) were used as experimental materials to explore the effects of length of sterile seedlings, size of stem nodes, genotype and hormone concentration on stem node regeneration. The experimental results showed that the optimal length of the sterile seedlings was 4.5–5.0 cm, with 1.0 cm the length of the stem node, the upper and lower sides retained 0.5 cm, the NAA hormone range varied between 0.3–0.5 mg L^{-1}, germination rate and seedling formation rate of the maize inbred line GSH9901 were the highest. Finally, the optimal value range of each factor was established, and the regeneration system of maize stem node was preliminarily established, which laid a foundation for further optimization of regeneration system and genetic transformation. © 2019 Friends Science Publishers

Key words: Maize; Regeneration system; Stem nodes

Introduction

Maize (*Zea mays* L.) belongs to the Poaceae family and is human important food and cash crops (Zhao *et al.,* 2018) with significant role in grain production of the world (Dowd *et al*., 2018). The great efforts are being carried out to improve the quality of maize exploring problems for a long time. Tissue culture is an important link of maize genetic transformation. With the rapid development of tissue culture technology, regenerated plants can be successfully obtained by using anthers (Ting *et al*., 1981), young embryos (Fromm and TavLor, 1986; Todorova *et al*., 1988; Yu *et al*., 2013), stem tips (Zhen *et al*., 2018), mature embryos (Zhao *et al*., 2008; Wang *et al*., 2012) and stem nodes (Shuren *et al*., 2005) as explants.

Jia *et al.* (2014) used the anthers of primrose as explants, and induced callus induction at appropriate developmental stages to obtain regenerated plants. Yuan *et al.* (1997) and others used the immature embryos of the conventional maize inbred lines. The implants were induced into callus and finally regenerated into seedlings. Li and Zhang (1999) successfully established the receptor system of corn shoot tips by tissue culture with stem tips as explants; Wang *et al.* (2008) used maize mature embryos as explant materials. Inducing embryogenic callus to obtain high-frequency regenerated plants; Armstrong *et al.* (2005) selected H99, LH198×HiII seeds, and after seed germination to seedlings for 7–10 days, half of the upper and lower parts were selected. The stem segments are inoculated in the medium for induction and finally regenerated into shoots.

Compared with other receptor materials, the regeneration system established by using stem node as explant reduced some complex engineering such as induction and differentiation (Du *et al.*, 2010). In this study, four factors including genotype, hormone concentration, stem-node size and the length of sterile seedlings were selected to study the influence of each factor on rooting and seedling survival rates of maize stem-node. The optimal level of each factor was preliminarily determined, laying a foundation for optimization of maize stem-node regeneration system and genetic transformation.

Materials and Methods

Test Materials

The seeds of four maize inbred lines (GSH9901, H4065, H4077 and H4051) were taken as raw materials and stored in the key laboratory of crop molecular breeding of Jilin province.

Medium

The basic medium used in the experiment was MS medium,

in which the germination medium and the rooting medium were followed Table 1

Methods

Seed Sterilization

Mature maize seeds with plump were selected and soaked in 70% ethanol in a sterile environment. After soaking for 5 min, the seeds were washed twice with double distilled water, soaked in 3% NaClO for 3 h, and washed 5-7 times with double distilled water. After sterilization of maize grain vertically inserted into the germination medium at 24°C in dark conditions.

Induction Treatment for Stem Node at different Concentration of NAA

From sterile seedlings, stem nodes were cut from the ultra-clean work, and placed on the rooting medium. The rooting medium was 1/2 MS as the basic, and 1-naphthlcetic acid (NAA) of different concentration was added to the medium Table 2.

Induction Treatment for Stem Node at different Lengths of Sterile Seedling

After sterilization, four lines of maize grain in preparation of good seed germination medium to dark, respectively is 0.5–0.6 cm long, 1 cm, 2–3 cm, 4.5–5 cm no vaccine, for the same size of internodes were inoculated to the rooting medium, bottle vaccination 25 stem section, repeated 4 times, 7 d after observation to take root and sprout.

Induction Treatment for Stem Node at different Lengths

Four inbred lines of sterilized maize seeds were inoculated on the prepared germination medium, and three stem segments of different sizes were cut when the length of the sterile seedlings was 4–4.5 cm:

(1) above the stem node was not retained, and the lower part of the stem node was kept 0.5 cm;

(2) 0.5 cm above stem node and 0.5 cm below stem node;

(3) 0.5 cm above the stem node and 0.25 cm below the stem node;

The cut stem segments were inoculated in rooting medium with NAA concentration of 0.3–0.5 mg L^{-1}. Twenty five stem segments were treated 4 times at a time; the root status and seedling status were observed at 7 d.

Induction Treatment for Stem Node at different Genotypes

Four kinds of sterilized maize seeds were inoculated in germination medium, and when the sterile seedlings grew to 4.5–5 cm, the length of stem segments was cut to 1 cm, the upper and lower parts were kept 0.5 cm respectively and the seedlings were cultured in rooting medium. Each inbred line treated 25 stem segments, repeated 4 times, the rooting and seedling formation of stem segments were observed 15 d later.

Table 1: The composition of the medium used in regeneration of maize

Medium	composition	pH
Germination medium	MS medium +500-600 mg L^{-1} casein +30 g L^{-1} sucrose +6.5 g L^{-1} agar	5.8
Rooting medium	MS medium +500-600 mg L^{-1} Casein+30 g L^{-1} Sucrose+6.5 g L^{-1} Agar+0.3-0.5 mg L^{-1} NAA	5.8

Table 2: The hormone concentration of the medium

Test number	NAA (mg. L^{-1})
1	0.1
2	0.3
3	0.5
4	1
5	2

Table 3: The shoot and root situation of the node

Retention length	Seedling rate after three days	Rooting rate after three days	One-week seedling rate	One-week root rate
(cm)	(%)	(%)	(%)	(%)
0 on the festival, 0.5 on the festival	0	65	0	100
0.5 on the festival, 0.5 on the festival	65	65	100	100
0.5 on the festival, 0.25 under the festival	65	30	100	85

Table 4: The regeneration situation of different genotypes maize node

Genotype	Regeneration rate (%)	Average number of items (a)
GSH9901	98	3.6
H4065	83	2.8
H4077	74	2.0
H4051	85	2.2

Table 5: Characteristics of two receptor systems

Factor	Stem nodes	Stem tip
Material	Maize sterile seedling	Maize sterile seedling
Operating	Easy to operate	Difficult to operate
Genotype restriction	NO	YES
Cycle length	Short	Short
Whether need to induce buds	NO	YES
Whether need to grow roots	YES	YES

Establishment of Maize Stem Node Regeneration System

Mature maize seeds were selected and soaked in 70% ethanol under aseptic conditions. After soaking for 5 min, the seeds were washed twice with double distilled water, and then soaked in 3% NaClO for 3 h. After sterilization of maize seed inoculation to the germination medium, were cultured at 24°C light conditions.

After germination, seeds were cultured under light. When the length of sterile seedlings reached 4.5–5 cm, stem segments were cut 0.5 cm above and below, respectively, and inoculated into rooting medium in a sterile environment. The rooting medium were cultured under light at 25°C for 3 weeks and observed regularly. The stem nodes grew distinct roots and leaves, and finally formed intact corn plants.

When the regenerated seedlings grow to a certain period, these were refined. During cultivation, the seedlings were in contact with air gradually, and some sterile water added to the culture joint to prevent the culture medium from being contaminated with bacteria.

Results

Effect of NAA Concentration on Stem Node Regeneration

The stem nodes of GSH9901 were inoculated in the medium of 1/2 MS without adding hormones. Although, the buds grew normally but roots were less-developed, with few fibrous and slightly thick roots (Fig. 3A). Grafting after seedling formation was not easy to survive. In this experiment, NAA with different concentration gradients was added into the rooting medium. The cut stem segments were inoculated into the rooting medium for rooting culture, and the rooting number and root length were counted 15 d later.

When the concentration of added NAA was less than 0.5 mg L^{-1}, the average rooting number and average root length showed an increasing trend with the gradual increase of NAA concentration (Fig. 1, 2). When NAA concentration was greater than 0.5 mg L^{-1}, the mean values of root number and root length showed a downward trend. When the concentration range of NAA was 0.3–0.5 mg L^{-1}, the average number of roots was the largest, about 2.4. Average length of the root was 2.1 cm, and the root was thick and long.

The root system of maize was whisker, and clumps of whisker root can be induced at the base of stem node to ensure the survival rate after transplanting. Root growth of maize stem segments was induced by rooting medium with different concentration gradient of NAA, which showed two different situations.

In the first case, the roots were short and thick with fewer fibrous roots (Fig. 3B). In the second case, the roots were thinner and shorter (Fig. 3A), which cannot guarantee the normal growth of the seedlings in the later stage. The lack of nutrition supply leads to the weak vitality of the seedlings (Fig. 3C). When NAA concentration ranged from 0.3–0.5 mg L^{-1}, fibrous roots were long and numerous (Fig. 3D) and the roots of the seedlings were developed and suitable for transplanting and had a high survival rate.

Effect of Length of Sterile Seedling on Stem Node Regeneration

When the length of germinated seedlings of maize seeds

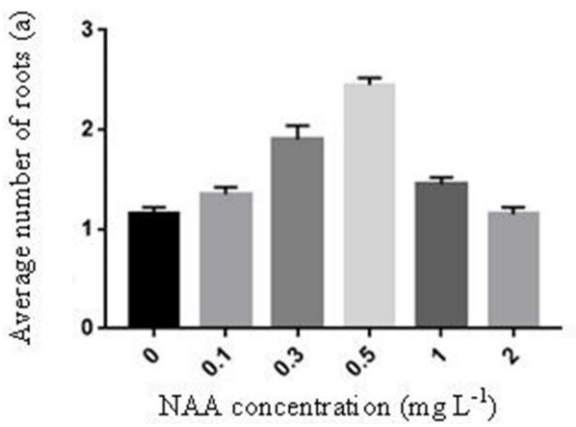

Fig. 1: Effects of NAA concentration on the rooting number of plantlets

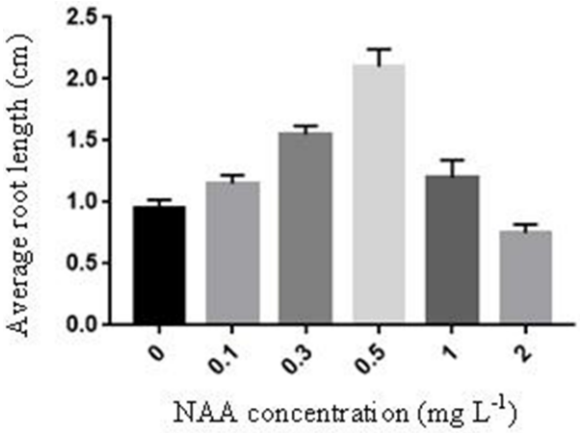

Fig. 2: Effects of NAA concentration on the rooting length of plantlets

was 0.5–0.6 cm, 1 cm, 2–3 cm, and 4.5–5 cm respectively, the seedling rate of stem nodes of the same size could be observed 1 week later after being cut and placed in rooting medium under the ultra-network. The seedling rate of stem nodes was the highest when length of seedlings of maize seeds was 4.5–5 cm (Fig. 4).

When the length of the sterile seedlings was 0.5–0.6 cm, 1 cm and 2–3 cm, the buds and roots do not grow well, and the roots have different degrees of protrusion, which may affect the normal growth of the stem nodes. When the growth length of cultured seedlings was 4.5–5 cm, the growth state of cut stem nodes was normal (Fig. 5).

Effect of Stem Size on Stem Node Regeneration

In this experiment, the upper and lower tissues of the stem node were cut from the protruding part of the stem node (Fig. 6). It can be seen that no tissue was retained in the upper part of the stem node, and no buds were grown 7 d later (Table 3). For tissue, 0.5 cm in the upper part of the stem node, the germination rate was 65% after 3 d and 100% after 7 d. The

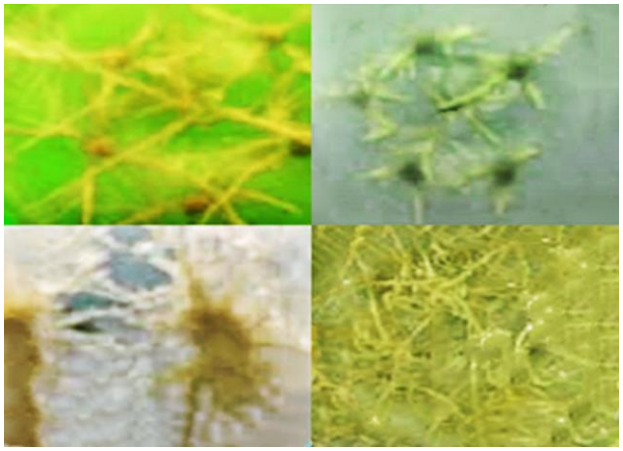

Fig. 3: The rooting situation of the maize node

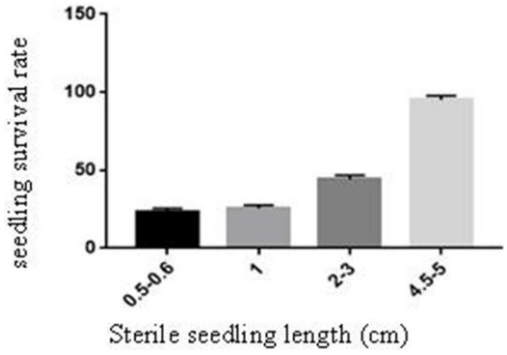

Fig. 4: Effects of sterile seedling length on seedling survival rate

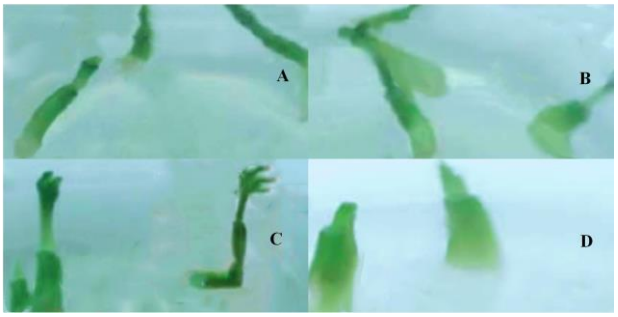

Fig. 5: The growth condition of maize node after a week
A: The state of the node after a week (0.5 -0.6 cm seedlings); **B**: The state of the node after a week (1 cm seedlings); **C**: The state of the node after a week (2-3 cm seedlings); **D**: The state of the node after a week (4.5-5 cm seedlings)

lower part of the stem node was kept 0.25 cm, and the roots were grown 30% after 3 d and 85% after 7 d (Fig. 7). The results of this experiment showed that the upper and lower parts of the stem nodes were kept 0.5 cm, and the rooting rate, budding rate and seedling rate were the highest.

Effect of Genotype on Stem Node Regeneration

Four maize inbred lines were used as materials, and the sterilized seeds were planted on the germination medium under the ultra-clean working table. The stem node regeneration rate of the four genotypes was GSH9901>H4051>H4065>H4077, and the maximum regeneration rate was 98% (Table 4). The regeneration rates of H4065 and H4051 were basically similar and the regeneration rates of H4077 were relatively low compared with the other three inbred lines with average number within this order GSH9901>H4065>H4051>H4077. Based on the above two conditions, it was concluded that the stem node of maize inbred line GSH9901 was the best material for stem node regeneration induction.

Establishment of a Stem Node Regeneration System of Maize

The stem segments of the four maize inbred lines were used as materials and the cut stem segments were directly seeded by induction to obtain regenerated plants. Fig. 8showed the process of regenerating plants in stem segments.

Discussion

At present, the improvement of maize quality is valued by many countries, and the main means of improvement is genetic engineering technology. The establishment of maize receptor system has become the key link of genetic transformation. With the continuous development of maize tissue culture technology, the immature embryo, anther and flower spike of maize can establish the receptor system and then regenerate into a complete plant. Immature embryo is the most commonly used explant, but its induction is also affected by many factors (Ge *et al*., 2017). In this experiment, the stem segments of mature seeds were used as materials. Compared with the callus culture of maize immature embryos, it was not restricted by seasons. As long as there were mature seeds, it was not restricted by genotype and the callus was removed to avoid complex work such as induction and differentiation.

In addition to induction of callus into seedlings, the success was obtained in tissue culture by using maize stem tips as explants. Maize stem tip is considered as an excellent material for the establishment of receptor system. In this experiment, the regeneration system of maize stem nodes was successfully established by using the stem nodes of four maize inbred lines as explants. Compared with the regeneration system established by the stem tips of maize, both of them had advantages and disadvantages (Table 5).

In addition to the basic components of germination culture and rooting culture media, acid hydrolyzed casein was added into the two media respectively. Di *et al.* (2011) took zheng 58 and Dan 598 as experimental materials and showed that acid-hydrolyzed casein would have an important effect on the germination of maize seeds which also evident from present study results. Di *et al.* (2011) found that the germination of seeds is affected by light.

Fig. 6: Schematic diagram of stem section

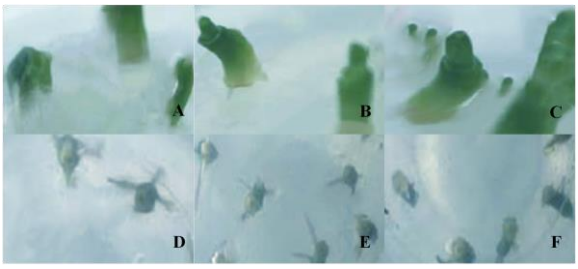

Fig. 7: The growth condition of the maize node after a week
A: emergence condition of 0 cm and lower 0.5 cm on the stem section of the maize; B: emergence condition of 0.5 cm and lower 0.5 cm on the stem section of the maize; C: emergence condition of 0.5 cm and 0.25 cm under the stem section of the maize; D: Roots of 0 cm and 0.5 cm below the stem section of the maize; E: roots of 0.5 cm and 0.5 cm below the stem section of the maize; F: roots of 0.5 cm and 0.25 cm below the stem section of the maize

Compared with dark culture, the seedling time of light culture were short and relatively strong. Therefore, in this experiment, when the seed germinated, the dark culture was not continued and the light culture was carried out by transfer, and the final seedling of the stem section had great benefits.

The growth environment of sterile seedlings is very superior, resulting in much weaker seedlings than grown in soil that need to refine. Seedling refining is a process in which sterile seedlings are gradually exposed to air, during which temperature and humidity should be paid attention to (Long and Li, 2006), and the medium should not be contaminated.

For higher survival rate, strong seedlings should be chosen before transplanting into hydroponic cultures. The advantage of hydroponic culture is that it can provide sufficient nutrients and continuous oxygen supply, so that the root system can grow quickly (Benzle and Cornish, 2017; Chen *et al.*, 2018). In this way, sufficient preparation is provided for the next transplanting, thus improving the survival rate after transplanting.

On the basis of previous studies, in order to ensure that the corn stem section can successfully obtain healthy regenerated plants, it was summarized the following suggestions during the experiment: the temperature and humidity during the post-transplanting process must be suitable. The optimum temperature range should be 25–30°C. If the temperature is high, the soil will easily breed bacteria; if the temperature is low, the plant will die due to the inability to withstand the cold. The optimum range of humidity is 60–70%. If the humidity is too small, the soil will be dry and not conducive to the growth of the plant; if the humidity is high, the roots of the plant will be too moist to breathe and die.

Conclusion

In this study, the stem sections of four maize inbred lines of GSH9901, H4065, H4077 and H4051 were used as experimental materials and the effects of length of sterile seedlings, size of stem sections, genotype and hormone concentration on stem regeneration were investigated. It was concluded that at 4.5–5 cm length of the sterile seedling, the seedling rate of the stem section is the highest; the upper and lower parts of the stem section were kept 0.5 cm, and the rooting rate, sprouting rate and seedling rate were the highest; according to the regeneration and average number of stems. The stem section of maize inbred line GSH9901 was preliminarily determined, and the rooting rate, germination rate and seedling rate were the highest. The range of NAA hormone was 0.3–0.5 mg L^{-1}, and the rooting rate of corn stem section was the best. Based on the above results, the maize stem section regeneration system was initially established, which provided an effective strategy for the subsequent genetic transformation of maize.

Acknowledgements

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