第29卷 第5期
2003年9月 664～669页
作 物 学 报
ACTA AGRONOMICA SINICA
Vol. 29, No. 5
pp. 664～669 Sept., 2003

# Regeneration Study of Soybean Cultivars and Their Susceptibility to *Agrobacterium tumifaciens* EHA 101

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**Abstract** The soybean transformation procedure included the *Agrobacterium*-cotyledonary node system and the bar gene as the selectable marker coupled with glufosinate as a selective agent. Cotyledonary nodes from 5 to 6 days germinated soybean seeds were used as explants. The explants, were wound by slicing 5 to 6 times, inoculated with *Agrobacterium tumefaciens* EHA 101 with pPTN140, then followed 3 days co-cultivation, washed the explants by wash medium which contain antibiotics, put explants onto shoot initiation medium with 5 mg/L glufosinate for selection. Regeneration rate of different soybean cultivars was counted 2 weeks later, and their susceptibility to *Agrobacterium tumefaciens* was investigated 4 weeks later by GUS assay. According to our experiments, Heinong 35, Zhongzuo 975(Zhonghuang 13), Hefeng 35, Zhongzuo962 are better than Thorne for regeneration, William 82, PI 361066, Heinong 35 and Zhongzuo 975 were better than Thorne for transformation.

**Key words** Soybean; Regeneration; Susceptibility to *Agrobacterium*

# 大豆品种的再生性能及对 EHA 101 农杆菌的敏感性

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**摘 要** 大豆转化可利用农杆菌和子叶节转化系统,*bar*基因作为选择标记,草丁膦作为选择试剂。用5-6 d发芽的种子的子叶节作外植体,在子叶节处划5-6下,用含pPTN 140的农杆菌 EHA 101感染后,共培养3 d,用含抗生素的洗液洗去外植体上的农杆菌,将外植体放入5 mg/L草丁膦的长芽培养基,两周后统计不同大豆品种的再生率,4周后做GUS染色对含pPTN140的农杆菌的敏感性进行统计。结果得知:黑农35、中作975、合丰35、Zhongzuo 962是在再生方面比Thorne更好的品种。William 82、黑农35、中作975、PI 361066转化频率较高。

**关键词** 大豆; 再生; 对农杆菌的敏感性
**中图分类号**: S565 **文献标识码**: A

Soybean is a very important crop in China and in the world. During the past 11 years the soybean production increased 58.7%. Combination of traditional breeding method with genetic engineering techniques for development of new cultivars with herbicide resistance, insect resistance or good seed quality is very important[1-4]. *Agrobacterium*-mediated transformation of soybean using the cotyledonary node as the explant for gene transfer was first achieved by Hinchee *et al*. (1988)[2]. Zhang *et al*. described the soybean *Agrobacterium*-mediated transformation system with bar gene as the selectable marker coupled with glufosinate as a selective agent[3]. Zhou *et al*. introduced *Bacillus thuringiensis* cryIA gene into soybean successfully with *Agrobacterium*-cotyledonary node transformation system[4]. Genes were also trans-

* **Foundation item**: This research was supported by Nobel Foundation in USA from 1999—2000 and 948 project from MOA, PRC.
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Received: 2002-11-08, Accepted: 2003-01-31.

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ferred to soybean protoplast by some researchers[5-6]. Soybean shoot organogenesis could occur from tissues such as cotyledonary nodes[7-9] and primary leaves of seedlings[10] while somatic embriogenesis occurred from immature embryos and cotyledons[11-12] of developing seeds.

*Agrobacterium*-mediated transformation was the best method available for DNA transfer to tissue explants. In order to produce soybean tumors with significant size, soybean cultivars and *Agrobacterium strains* had been screened to find the optimal compatible response. Successful transformation of *in vitro* soybeans leaves, cotyledons, and protoplasts[13,14] were demonstrated.

*Agrobacterium* was used as the biological vector to introduce a portion of its DNA into the plant genome, resulting in production of transformed plants. Cotyledonary nodes were wounded and inoculated with *Agrobacterium*. The wounded plant tissues gave off specific phenolic compounds which induce *Agrobacterium* to express a set of *vir* genes. *vir* genes is responsible for the excision and transfer of the T strand from the bacterium into the recipient plant cell.

*bar* gene encodes for phosphinothricin acetyltransferase (PAT) which detoxifies glufosinate. Glufosinate is the active ingredient in the herbicide Liberty. Kanamycin was used as selective agent in previous researches. In this paper glufosinate was used as selective agent.

However, there is still some degree of cultivar specificity of transformation efficiency (Hinchee *et al*., 1988)[2]. And to study this cultivar specificity is the main task of this research.

## 1 Material and Methods

### 1.1 Material and Seed Sterilization

3 to 4 varieties were evaluated each experiment, Thorne were used as control. To total 15 cultivars such as Zhongzuo 975, Zhongzuo 962, Zhongzuo 966, Zhongzuo 965, Zhongzuo M17, Zhonghuang 4, Heinong 35, Heinong 37, Hefeng 35, NE3297, William 82, U96-2208, PI 361066, A3237 and Thorne were screened for good regeneration and susceptibility to EHA 101 with pPTN140. The seeds were surface sterilized by two days exposure to chlorine gas by mixing 100 mL of a 5.2% sodium hypochlorite (Chlorox bleach) with 3.3 mL of 12 mol/L HCl. The procedure should be conducted within a fume hood. The seeds are ready for the germination step[15-25].

### 1.2 Soybean germination

Sterilized seeds were germinated in 100 mm × 20 mm Petri dishes on $B_5$ medium supplemented with 2% sucrose, pH 5.8. The plates stacked 5 high and placed in plastic bags, 5 small holes were made by scissors. Seed were for five days in a growth room at 24 ℃, 18/6 light regime, or for 6 days (at 5th day put the seeds to 4 ℃ refrigerator).

### 1.3 Plant transformation vectors

EHA101 containing binary vector pPTN140 were used, and the resistances to 25 mg/L chloramphenicol [EHA 101 chromosomal drug marker], 50 mg/L kanamycin [Ti plasmid pEHA 101 drug marker], 100mg/L spectinomycin [binary vector drug marker] and 100 mg/L streptomycin [binary vector drug marker] were included in the *Agrobacterium* and vector[3].

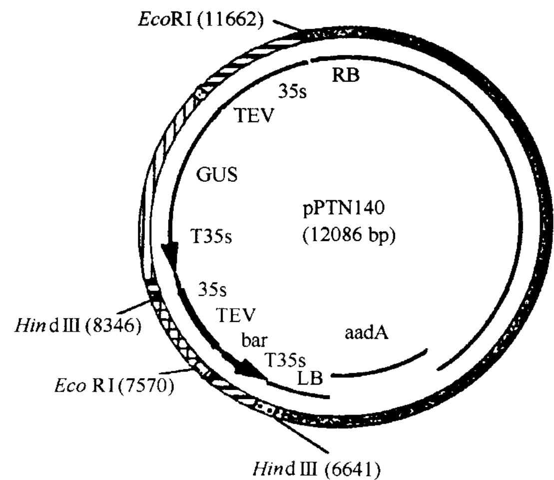


Fig. 1 Binary vector utilized in the experiments
Abbreviations: RB-right border, LB-left border, T35s and pAg7 are polyadenylation signals, Pnos-nopaline synthase promoter, aadA-bacterial drug resistance marker for spectinomycin and streptomycin.

### 1.4 Preparation of Agrobacterium

*Agrobacterium* was streaked from frozen glycerol stocks onto solid YEP medium with appropriate antibiotics, and grew for 3 days. One day before co-cultivation 2 mL *Agrobacterium* were cultured in 10—15 mL tube for about 10 to 12h, then subcultured 0.5 mL *Agrobacterium* to 150 mL to 250 mL with appropriate antibiotics for about 12 to 16 h until the $OD_{650}$ = 1.1 to 1.2 at 27 ℃. The bacterial cultures were centrifuged then at 3,500 r/min for 10 min, and the pellet was resuspended to a final $OD_{650}$ = 0.6 - 0.8 in 1/10 Gamborg's $B_5$ medium amended with 1.67 mg/L BAP, 0.25 mg/L $GA_3$,

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200 μmol/L Acetosyringone (AS) and 3 % sucrose. The medium was buffered with 20 mmol/L MES, pH 5.4. All growth regulators, vitamin components and AS were filter sterilized by using syringe after autoclaving.

**1.5 Plant transformation**

1.5.1 Explant slicing

Green seeds were only chosen from the 5-day or 6-day old soybean seedlings and cotyledonary explants were prepared for making a horizontal slice on the hypocotyl region. The embryonic axis was removed, about 5 —6 vertical slice was made on the adaxial surface of the explant at the cotyledon and hypocotyl junction by blade. The slicing was 0.5 mm deep and 3 —4 mm long.

1.5.2 Inoculation and Co-cultivation

Co-cultivation medium contained 1/10 the salts and vitamins of Gamborg's medium amended with 1.76 mg/L BAP, 0.25 mg/L $GA_3$, 200 μmol/L AS, 3 % sucrose and 20 mmol/L MES buffer, pH 5.4 (Note: filter sterilize all growth regulators and AS), and it should be prepared fresh the day before the inoculation of soybean explants.

Explants were put in *Agrobacterium* solution for 30 min to 1 h, and then on 100 mm ×15 mm Petri plates containing the co-cultivation medium solidified with 0.5 % purified agar. The co-cultivation plates were overlaid with a piece of Whatman # 1 filter paper. The explants (5 per plate) were placed adaxial side down on the co-cultivation plates for 3 days at 24 ℃, under an 18/6 h light regume.

1.5.3 Wash and shoot initiation

After the co-cultivation period the explants were briefly washed with $B_5$ medium containing 1.67 mg/L BAP, 3 % sucrose, 50 mg/L ticarcillin 50 mg/L cefotaxime and 50 mg/L vancomycin. The medium was buffered with 3 mmol/L MES, pH 5.6. Growth regulator, vitamins and antibiotics were filter sterilized by using syringe after autoclaving. After the washing step, explants were cultured in 100 mm ×20 mm Petri plates, adaxial side up with the hypocotyl imbedded in the medium, containing the washing medium solidified with 0.8 % purified agar amended with 4 or 5 mg/L glufosinate. This medium was referred as shoot initiation medium (SI). The explants were cultured under the same conditions as the seed germination.

1.5.4 Regeneration rate

After 2 weeks of culture, regeneration rate was counted by the ratio of regular shoot and total explants set up. Regular shoots referred to a lot of shoots regenerated from cotyledonary node. If on the middle of cotyledonary node a big shoot was growing, this kind of shoots referred to axillary shoot. Axillary shoot occurrence was because the explant didn't wound enough. No regeneration referred to nothing growing on the cotyledonary node, this was because the explant wounded too much.

The bottom of the hypocotyl region was excised from each of the explant. The regular explants, cotyledon with differentiation nodes were subsequently subcultured on fresh SI medium.

1.5.5 GUS assay

After 4 weeks of culture, histochemical GUS assay was carried out to see how many GUS sectors and how many GUS differentiating shoots were developed. Soybean tissues were vertically sacrificed on differentiating region for 5 to 6 times and incubated in the X-Gluc substrate plus GUS assay solution for 8 h at 37 ℃, then taking the solution away, storing the tissues in 70 % ethanol prior to observation under microscope.

1.5.6 Shoot elongation

The cotyledonaries were cut away from the differentiating shoots. The multiple shoots were subcultured on shoot elongation medium (SE) composed of Murashige and Skoog (MS) (1962) basal salts, $B_5$ vitamins, 1 mg/L ZR, 0.5 mg/L $GA_3$ and 0.1 mg/L IAA, 3 % sucrose and 3 mmol/L MES, pH 5.6. The SE medium was amended with 3 mg/L glufosinate.

1.5.7 Rooting

When reached 3 cm, the shoots were cut at the bottom and rooted on MS salts with $B_5$ vitamins, 1 % sucrose, 0.5 mg /L NAA without further selection in Sundae cups (Industrial Soap Company. St Louis MO).

1.5.8 Transfer to soil

When the roots reached 0.5 - 1.0 cm, the plantlet were transferred onto soil after carefully washing with water.

## 2 Results

After putting the explants into SI medium for 2 weeks regeneration rate was checked. Experiment 1 showed that 62 explants of Zhongzuo 975 were set up, 51 explants with good regeneration. Another 2 weeks later, the number of GUS sectors, buds and shoots calculated. GUS sector referred to GUS expression bigger than 1mm in size. Several GUS sectors

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could be obtained from one explant since one sector might be sacrificed several times. Buds or shoots were observed from 62 explants in Zhongzuo 975. GUS differentiation shoots referred to GUS expression, either chimeric or clonal, within differentiating structures with leaf development. Susceptibility to *Agrobacterium tumefaciens* EHA 101 depended on GUS assay results. The more GUS differentiating shoots showed, the better susceptibility to EHA 101 was. Good experiments should show at least 4 % GUS + bud/shoots and 40 % GUS + sector among differentiation and nondifferentiation tissues, respectively.[16-25]

So as a result of experiment 1, Heinong 35 and Zhongzuo 975 gave better regeneration rate and better susceptibility than Thorne. The regeneration rate of Heinong 35 exceeded 17.29 % over Thorne.

In experiment 2 of Zhongzuo 975, Zhongzuo 962 and Thorne used, among 124 explants of Zhongzuo 975, 109 explants produced regular shoots, 30 explants gave GUS sector, 6 explants showed GUS differentiating shoots. Of 91 explants from Thorne, 60 became regular shoot, 1 explant showed GUS differentiation shoot. So as a result of experiment 1 and 2, regeneration rate of Zhongzuo 975 exceeded Thorne by 14.02 %. Regeneration rate of Zhongzuo 962 exceeded Thorne by 8.36 %. It was suggested Zhongzuo 975 be better susceptibility than Thorne.

In experiment 3, regeneration rate of Hefeng 35 exceeded Thorne by 9.67 %, Hefeng 35 was more susceptibility than Thorne or Heinong 37.

**Table 1 Regeneration rate and GUS assays under 5 mg/L glufosinate selection**

| Experiment | Variety | Number of explants set up | Regeneration rate after putting explants onto SI medium for 2 wk | GUS assays after putting explants onto SI medium for 4 weeks | |
|---|---|---|---|---|---|
| | | | | GUS sector | GUS differentiating shoot |
| Exp 1 | Zhongzuo 975 | 62 | 51/62 (82.26 %) | 99 | 10 (16.13 %) |
| | Heinong 35 | 92 | 86/92 (93.48 %) | 61 | 11 (11.96 %) |
| | Zhongzuo 962 | 147 | 116/147 (78.91 %) | 2 | 0 |
| | Thorne (CK) | 44 | 32/44 (76.19 %) | 17 | 5 (11.36 %) |
| Exp 2 | Zhongzuo 975 | 124 | 109/124 (87.90 %) | 30 | 6 (4.84 %) |
| | Zhongzuo 962 | 175 | 130/175 (74.29 %) | 12 | 2 (1.14 %) |
| | Thorne (CK) | 91 | 60/91 (65.93 %) | 26 | 1 (1.10 %) |
| Exp 3 | Hefeng 35 | 68 | 58/68 (85.29 %) | 30 | 5 (7.35 %) |
| | Heinong 37 | 74 | 40/74 (54.05 %) | 17 | 1 (1.35 %) |
| | Thorne (CK) | 18 | 13/18 (72.22 %) | 0 | 1 (5.56 %) |
| Exp 4 | Hefeng 35 | 73 | 65/73 (89.04 %) | 150 | 3 (4.11 %) |
| | Heinong 37 | 59 | 46/59 (77.97 %) | 92 | 5 (5.43 %) |
| | Thorne | 9 | 8/9 (88.89 %) | 15 | 0 |
| Exp 5 | Heinong 37 | 73 | 55/73 (75.34 %) | 139 | 9 (12.33 %) |
| | Hefeng 35 | 74 | 62/74 (83.78 %) | 87 | 4 (5.40 %) |
| | Thorne | 50 | 34/50 (68.00 %) | 44 | 2 (4.00 %) |
| Exp 6 | William 82 | 30 | 18/30 (60.00 %) | 7 | 6 (20.00 %) |
| | U96-2208 | 183 | 136/183 (74.32 %) | 56 | 8 (4.37 %) |
| | Thorne (CK) | 87 | 65/87 (74.71 %) | 14 | 3 (3.45 %) |
| Exp 7 | Zhonghuang 4 | 125 | 92/125 (73.60 %) | 46 | 1 (0.80 %) |
| | Zhongzuo 966 | 33 | 23/33 (69.70 %) | 11 | 0 |
| | Thorne | 80 | 59/80 (73.75 %) | 14 | 3 (3.75 %) |
| Exp 8 | Zhongzuo M17 | 18 | 14/18 (77.78 %) | 5 | 2 (11.11 %) |
| | Zhongzuo M965 | 20 | 12/20 (60.00 %) | 13 | 2 (10.00 %) |
| | Thorne | 33 | 28/33 (84.85 %) | 21 | 0 |
| Exp 9 | NE 3297 | 61 | 49/61 (80.33 %) | 16 | 1 (1.64 %) |
| | Thorne | 70 | 64/70 (91.43 %) | 9 | 0 |
| Exp 10 | PI 361066 | 129 | 119/129 (92.25 %) | 138 | 16 (12.40 %) |
| | Thorne | 104 | 92/104 (88.47 %) | 29 | 4 (3.85 %) |

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In experiment 4, Hefeng 35 had higher regeneration rate and more susceptibility than control.

In experiment 5, Heinong 37, Hefeng 35 had higher regeneration rate and more susceptibility than Thorne.

In experiment 6, William 82 was more susceptibility than U96-2208 or Thorne.

In experiment 7, Thorne was more susceptibility than Zhonghuang 4 and Zhongzuo 966.

In experiment 8, Zhongzuo M17 and Zhongzuo M965 was more susceptibility than Thorne.

In experiment 9, NE 3297 was more susceptibility than Thorne.

In experiment 10, regeneration rate of PI 361066 exceeded Thorne by 3.78 %, and PI 361066 was more susceptibility than Thorne.

As the result of GUS differentiating shoot assay William 82 was the best cultivar for transformation, with 20 % of GUS differentiating shoots, PI 361066 with 12.40 %, Heinong 35 with 11.96 %, Zhongzuo 975 with 10.48 %. All of them were better than control Thorne.

## 3 Discussion

It is still a difficult event for most institutions in China to transform soybean routinely using *Agrobacterium* mediated transformation system.

**3.1** Choices of *Agrobacterium tumefaciens* strains and vectors, were proven to be critical. *Agrobacterium tumefaciens* EHA101 was an ideal strain for soybean transformation.

**3.2** 13 soybean cultivars had been tested, and regeneration rates of 5 cultivars were better than control. Cultivars whose regeneration rate exceeded control by 10 % were Heinong 35, Zhongzuo 975, Hefeng 35, Zhongzuo 962, PI 361066. The regeneration rate of other 8 cultivars didn't exceed control. Compared with Thorne, Heinong 35, Zhongzuo 975 (Zhonghuang 13), Hefeng 35, Zhongzuo 962 and PI 361066 were best genotypes for regeneration. However, more soybean cultivars need to be screened for higher transformation efficiency.[22-25]

**3.3** *Agrobacterium* mediated soybean cotyledonary node transformation can result directly shoot organogenesis, in which bar gene can be used as selectable marker.

**3.4** The best combination of co-cultivation was 24 ℃ temperature, 1/10 strength of $B_5$ salt and addition of 200 μmol/L acetosyringone.

**3.5** Satisfactory wounding during explant preparation played an essential role. Wounding influenced not only shoot differentiation but also *Agrobacterium* infection. We hope to get more regular shoots of regeneration instead of axillary shoots or non-regenerated shoots. If the explant was not wound enough, axillary shoots (one big shoot grows) would grow. If the explant was wound too much, no shoot might grow, and this should be regarded as non-regenerated shoot.

**3.6** Herbicide selection schemes needed to be optimal. Glufosinate can be used as the selective agent for bar gene. Typical problems associated with low selection pressure were the increased escapes, chimerism events. While the problems from high selection pressure were subsequent failure of transgenic recovery. Under standard culture conditions 5 mg/L glufosinate during shoot initiation period and 2.5 mg/L during shoot elongation were optimal.

**3.7** Antibiotics such as ticarcillin, cefotaxime and vancomycin were helpful for satisfactory results. If some of them were not available, Carbenicillin 300 — 500 mg/L plus some of above available antibiotics can be used as replacement.

**3.8** Use of L-cysteine as a strong antioxidant might enhance *Agrobacterium* infection on soybean substantially according to some research. The recommended concentration was 400 mg/L in co-cultivation medium.

**Acknowledgements**

This work was mostly conducted at the Plant Transformation Core Research Facility, Center for Biotechnology of the University of Nebraska-Lincoln. I'd like to express sincere thanks to my advisor Dr. T Clemente for his help and financial Support from Nobel Foundation. Some works conducted at Soybean laboratory of Institute of Crop Breeding and Cultivation Institute, Chinese Academy of Agricultural Sciences. Thanks to Prof. XIN Zhi-Yong and all the colleagues at the soybean department. Thanks to Prof. Samuel S Sun from Chinese University of Hong Kong, Dr Zhangyuan Zhang from Missouri University and SHAO Qi-Quan from Institute of Genetics, Chinese Academy of Sciences for their help and support.

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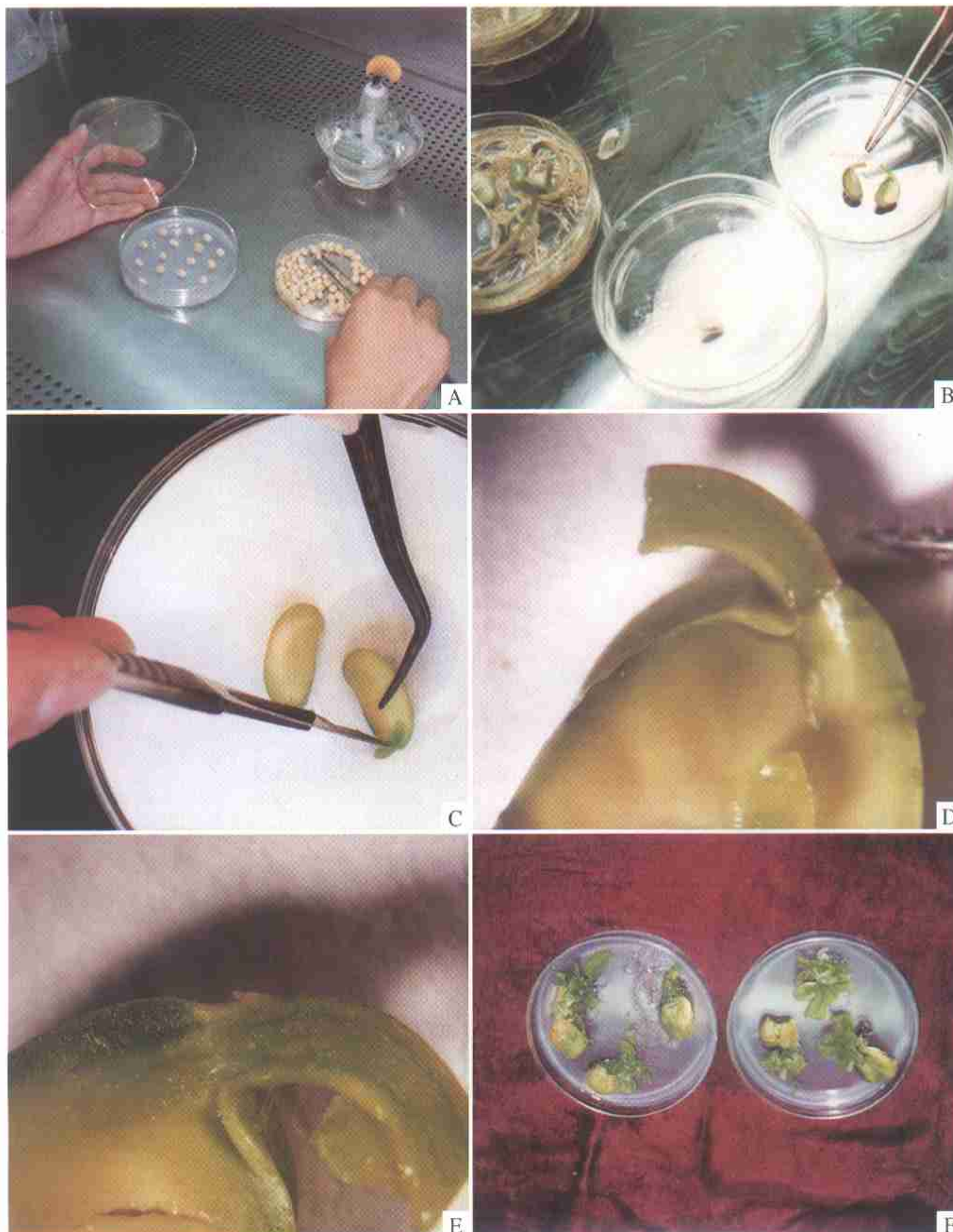


A: Sterilized germinated on B5 medium. B: Cotyledonary explants put in the *Agroba*. C: How to slice the explants. D: Embryonic axis was removed. E: Vertical slice on cotyledonary node through the hypocotyl region about 5 - 7 times. F: Shoot initiation of Zhongzuo 975.

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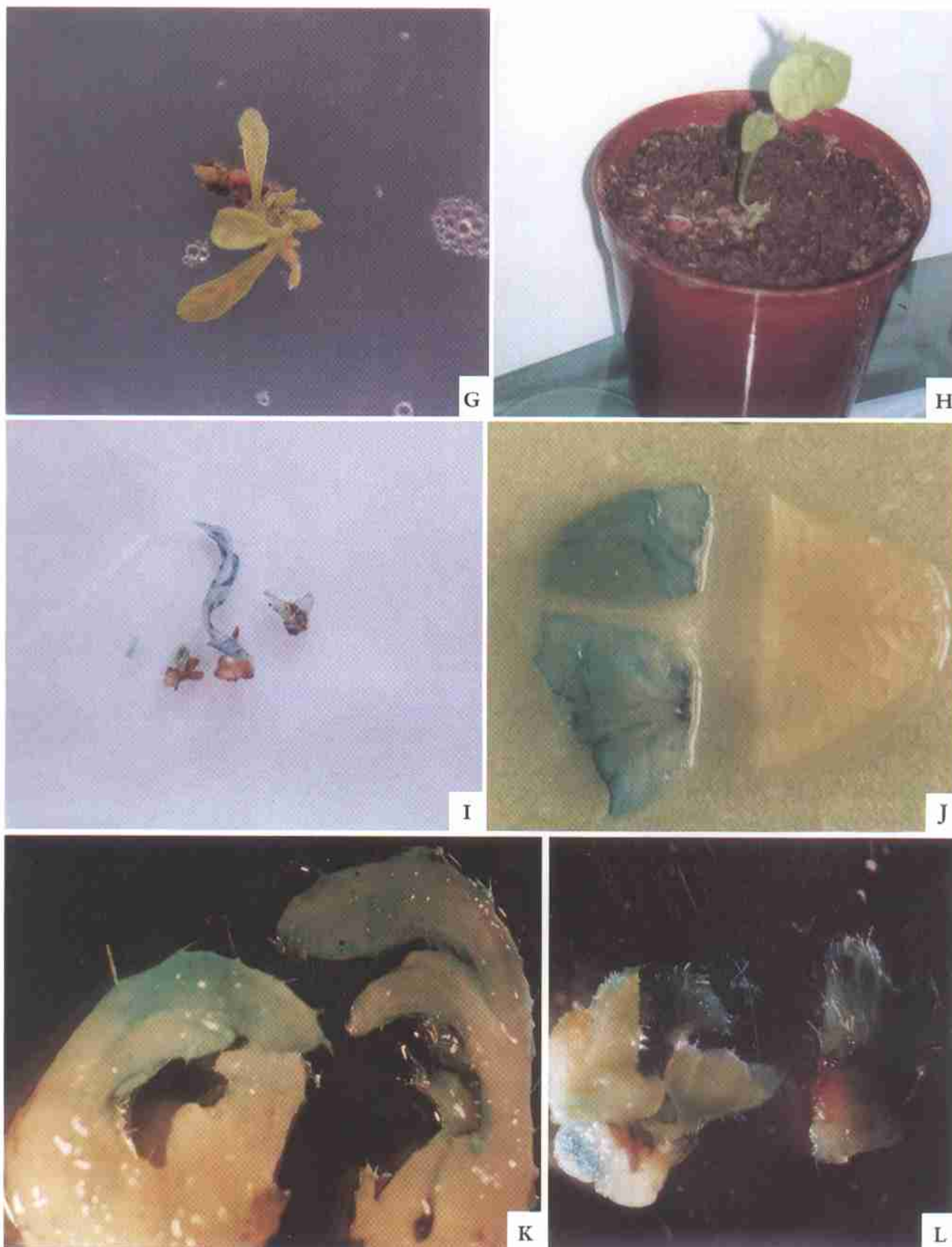


G: Shoot elongation of Heinong 35. H: Shoot reached 3 cm, the root reached 0.5 - 1.0 cm, the plantlet can be transfer to soil. I: Gus assay result of William 82. J: Gus assay of $T_1$ (Left) and $T_0$ (right). K: Gus assay result of William 82. L: Gus assay result of Heinong 35.

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