THAI NGUYEN UNIVERSITY
**UNIVERSITY OF EDUCATION**

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# STUDY ON TRANSFERRING *codA* GENE TO IMPROVE DROUGHT TOLERANCE OF SOYBEAN (*Glycine max* (L.) Merrill)

**Major: Genetics**
**Code: 9420121**

**SUMMARY OF BIOLOGY DOCTORAL DISSERTATION**

**THAI NGUYEN - 2021**

The dissertation was finished at
University of Education - Thai Nguyen University.

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The dissertation will be defended in the university committee:
University of Education - Thai Nguyen University
At … / … , … , …, 2021

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# PREFACE

## 1. Introduction

Climate change and sea level rise are taking place on a global scale, leading to droughts, especially prolonged salinity, causing great difficulties for the agricultural industry. Increased drought stress has become a major impediment, severely affecting global agricultural productivity and yield.

Soybean is one of the important agricultural crops in the world, providing nutritious protein at low cost and playing a vital role in soil improvement in agriculture. Soybean is one of the low drought-tolerant crops. Drought stress is the main cause of reduced yield and productivity of soybean. In the current context, improving drought tolerance in soybean to minimize yield loss in water-deficient conditions is an urgent task of agricultural breeders.

The improvement of drought tolerance of soybean has been studied with many different approaches, among which transgenic techniques have offered great prospects in improving the drought tolerance of soybean. Drought tolerance is a complex quantitative trait, influenced by a set of target genes. The gene expression directly affects the drought tolerance trait or regulates the function of the drought-tolerant gene group. One of the current research directions to approach the drought tolerance mechanism is to increase the substances that help protect the osmosis pressure of the cells from water imbalance. Among them, glycine betaine (GB) is one of the most widely studied osmotic protectants. Some transgenic plants with encoded enzymes involved in GB biosynthesis also show tolerance to adverse environmental conditions such as heat tolerance, cold tolerance, drought tolerance and salt tolerance.

The method of transferring *codA* gene from *A. globiformis* into crops such as green beans, potatoes, tomatoes, etc. to improve their tolerance under various abiotic stress conditions has been proven to be simple and effective. fruit. In Vietnam, the *codA* gene encoding GB biosynthesis has been successfully transferred into chinaberry and tobacco, showing drought and salt tolerance. Therefore, the study of enhancing *codA* gene expression related to GB biosynthesis to improve drought tolerance in soybean which might help plants grow better, giving higher yield and productivity in adverse environmental conditions is practical and necessary.

Stemming from the scientific basis and practical need for creating drought-tolerant soybean varieties by transgenic technology, we chose to carry out the

thesis titled “Study on transferring *codA* gene to improve drought tolerance of soybean *(Glycine max* (L.) Merrill)”.

**2. Aim of the research**

Transforming the *codA* transgene into soybean and creating a *codA* transgenic soybean that encodes choline oxidase which would be more drought tolerant than the non-transgenic plant.

**3. Content of the research**

3.1. Research on creating transgenic structures carrying the *codA* gene in plant expression vectors

1) Designing a plant gene expression vector containing a *codA* gene carrier.

2) Transforming transgenic structure carrying the *codA* gene into tobacco tissues. Evaluating transformed *codA* gene activities and expressions in transgenic tobacco varieties.

3.2. Research on the influence of some factors on the efficiency of *codA* gene transferred in soybean

1) Researching on the effect of phosphinothricin (ppt) concentration on the ability to create shoots from soybean cotyledons.

2) Analyzing the effect of bacterial concentration and incubation time, co-culture on the ability to induce soybean shoot formation.

3) Analyzing the effect of antibiotic concentration and bacterial cleaning time on the emergence and growth of soybean shoots.

3.3. Research on transforming structures carrying *codA* gene into soybean variety DT22 and creating drought-tolerant transgenic soybean plants

1) Genetic transforming and generating *codA* transgenic soybean plants.

2) Analyzing *codA* transgenic soybean plants.

3) Evaluating drought tolerance ability of several varieties of *codA* transgenic soybean plants.

**4. New contributions of the thesis**

The thesis is the first research work in Vietnam to successfully create *codA* gene-carrying soybean plants to increase drought tolerance compared to non-*codA*-gene-carrying soybean plants. The thesis is a systematic work with contents presented from plant transgenic vector design, gene expression analysis and *codA* transgenic plant with high cumulative GB content generation.

More specifically:

1) The appropriate factors for transferring *codA* gene and forming multi-shoot in soybean variety DT22 have been identified. PPT concentration of 3 mg/l at shoot induction stage in SIM medium and PPT concentration 1.5 mg/l at shoot elongation stage in SEM medium; bacteriophage with the value $OD_{650}$= 0,6, incubation time of 30 minutes, co-culture for 5 days in the dark and sterilization with cefotaxime 500 mg/l are suitable for shoot induction and shoot elongation on selective media.

2) For the first time, the *codA* gene was successfully transferred and strongly expressed in soybean in Vietnam. The expression of *codA* transgene in transgenic soybean plants increased GB, proline, POD activity, and decreased MDA content compared with non-transgenic soybean plants.

3) Four *codA* transgenic soybean varieties in the generation $T_1$ were evaluated, GB content in transgenic soybean varieties increased from 248.9% - 288.3% compared with non-transgenic plants; proline content increased 1.5-2 times, POD activity increased 4 times and MDA content decreased 0.5 times compared with non-transgenic plants.

**5. Scientific and practical significance of the thesis topic**

The research results obtained in the thesis have scientific and practical values in further research into improving the tolerance to abiotic stresses of soybean by transgenic techniques.

Scientifically, the results of the thesis have demonstrated that increasing the expression of *codA* gene with encoding the key enzyme in GB biosynthesis pathway has increased drought tolerance in soybean. The research results are the scientific basis for improving the tolerance to adverse environmental factors in plants in general and legumes in particular by gene expression techniques. Results published in scientific articles are valuable references for biology research and teaching.

Practically, *codA* transgenic soybean varieties can be used as materials for selecting drought-tolerant soybean varieties. The research results of the thesis can be applied to legumes and other plant species in the direction of research which aims at improving GB content to increase tolerance to adverse environmental conditions.

**6. Structure of the thesis**

The thesis has 134 pages (including appendix), divided into chapters and sections: Introduction (5 pages); Chapter 1: Literature review (40 pages);

Chapter 2: Research materials and methods (16 pages); Chapter 3: Results and discussion (39 pages); Conclusion and recommendations (1 page); Published works related to the thesis (1 page); References (23 pages); Appendix (10 pages). The thesis has 8 tables, 26 figures, 3 appendices and 180 references.

## Chapter 1. LITERATURE REVIEW

The thesis has consulted and summarized 180 documents, including 11 documents in Vietnamese, 169 documents in English on three main issues which are: (1) Impacts of drought and drought tolerance mechanism in plants; (2) GB, choline oxidase; (3) Improving drought tolerance in soybean by transgenic technology.

Drought stress affects the morphology, physiology and growth and has serious consequences in the flowering, seedling and fruiting, leading to 73–82% reduction in seeds/soybean plant. Drought stress causes dysfunction of the photosynthetic apparatus, reducing chlorophyll, thereby reducing the efficiency of photosynthesis. Drought stress strengthens the antioxidant apparatus: eliminates ROS radicals, reduces electrolyte leakage and lipid peroxidation to help maintain vitality, integrity of organelles and cell membranes. In addition, drought stress also reduces the ability of roots to absorb minerals in the soil, reduces the rate of mineral transport in the stem, leading to a decrease in ion content in the tissues of the plant body. Under drought conditions, plants often actively maintain physiological water balance in the following ways: (i) Enhancing root development and close stomata to reduce water loss. (ii) Enhancing solute accumulation and regulate osmotic pressure in tissues. (iii) Enhancing antioxidant capacity. (iv) Enhancing the regulation of hormones.

GB is a fully N-methyl substituted derivative of glycine. GB is a low-molecular-weight, quaternary ammonium compound, dipole, and electrically neutral at physiological pH. GB is a non-toxic, colorless, tasteless and odorless compound that is accumulated in many plant species. The degree of accumulation of GB is not the same in different species, different organs in a plant body. GB is a compatible penetrant, osmotic protectant or solubilizer. GB is considered a bio stimulant, used at low concentrations to promote plant growth, increase tolerance to abiotic stress, and improve yield. GB is synthesized in many ways, but the most common one is the secondary way starting with choline. There are 2 oxidation steps in plants: (i) choline is converted to betaine aldehyde, catalyzed by choline monooxygenase (CMO), then (ii) betaine aldehyde is

converted to GB by the catalysis of betaine aldehyde dehydrogenase (BADH). In animals and many bacteria, the first step uses choline dehydrogenase (CHDH) while in the second oxidation step the enzyme is still BADH. Bacteria *A. globiformis* and *A. pascens* only use choline oxidase (CHO) enzyme encoded by a single gene *codA* to catalyze the direct four-electron oxidation of choline to GB. Observing the biosynthetic ways of GB in different organisms, it can be seen that the biosynthetic way of GB in *A. globiformis* and *A. panescens* is the simplest. To create GB from the precursor choline, it is only necessary to use the enzyme choline oxidase (COD) which is encoded by the *codA* gene. Therefore, the *codA* gene encoding choline oxidase has become the target of biotechnological research to improve resistance to osmotic stress.

Choline oxidase from *A. globiformis* was first purified by Ikuta in 1977. This enzyme, a flavoprotein, catalyses the four-electron oxidation of choline to GB with the intermediate product being betaine aldehyde and the final electron acceptor being molecular oxygen. Specifically, in step 1, choline is oxidized to betaine aldehyde, $O_2$ is reduced to hydro peroxide ($H_2O_2$); in step 2, betaine aldehyde binds to the hydrated enzyme to give gem-diol choline, which is then oxidized to GB. The *codA* gene encoding choline oxidase of bacteria *A. globiformis* (GenBank, code AY304485) is 1641 bp in size and encodes a 547 aa polypeptide, saturated by G + C content, which interferes with the conduction of the polymerase chain reaction because its hairpin structure is resistant to melting. Therefore, it is necessary to design the genetic code and artificially synthesize the *codA* gene in accordance with the gene expression in the plant cell system (with excess A + T content). In addition, a 216 bp transit peptide-TP nucleotide sequence was added to the 5' end of the *codA* sequence (which encodes the tobacco Rubisco subunit transport protein) to transport the enzyme into the chloroplast. The 3' end of the *codA* artificial gene, supplemented with a 30 bp nucleotide fragment encoding the cmyc antigen, was used to test for the presence of recombinant *codA* protein in transgenic plants by western blot. Thus, the size of the artificially synthesized *codA* gene is 1887 bp (216 bp TP+ 1641 bp *codA*+ 30 bp *cmyc*). The function of choline oxidase encoded by the artificially synthesized *codA* gene was not altered from the original sequence.

Soybean is a low-cost source of nutritious protein, used as food for humans and livestock and a soil amendment crop. Soybean has a short growth period, wide adaptability, and it can be arranged to suit many crop structures in

production, but it belongs to the group of crops with poor drought tolerance. Dr Drought reduces germination, leading to poor growth, reducing photosynthesis and mineral absorption. Therefore, improving drought tolerance in soybean plants to minimize yield losses when there is a lack of water is an urgent task of agricultural plant breeders.

Transgenic soybean is the first GM crop to enter mass production, with the largest planted area in the world. The *Agrobacterium* gene transfer technique is the most widely used. In the world and in Vietnam, many studies have been successful in creating transgenic soybeans which are more tolerant to abiotic stress. Many studies have been carried out on transgenic *codA* gene encoding choline oxidase GB biosynthesis to increase the resistance of plants to adverse environmental conditions from the outside. However, there are currently no published studies on *codA* gene transfer in soybean. Therefore, the transfer of *codA* gene into soybean to improve drought tolerance is a new, timely and urgent direction.

## Chapter 2. RESEARCH MATERIALS AND METHODS

### 2.1. RESEARCH MATERIALS

***Plant materials:*** Tobacco variety K326 provided by Tobacco Research Institute. Soybean variety DT22 provided by the Bean Research and Development Center.

***Bacterial strains and vectors:*** *E. coli* strains DH5α and *A. tumefaciens* C58/pGV2206. Vector *pIBTII, pCAMBIA* were designed and used at the Department of Plant Cell Technology, Institute of Biotechnology, Vietnam Academy of Science and Technology. The *codA* gene sequence encoding for choline oxidase from *A. globiformis* mined on Genbank (Code AY304485) optimized for expression in plants was provided by Epoch Life Science Inc, USA.

***Primer pairs used for PCR reaction:*** *codA-F/R, Bar-F/R, F/TP-XbaI, R/cmyc-SacI.*

### 2.2. CHEMICALS, EQUIPMENT

***Chemicals:*** Chemicals of Fermentas, Invitrogen, Sigma, Merck, Amersham Pharmacia Biotech, etc.

***Equipment:*** Equipment of Department of Plant Cell Technology and Gene Technology Key Laboratory, Institute of Biotechnology.

### 2.3. METHODOLOGY

#### 2.3.1. Methods of designing vectors of transgenic *codA* and transgenes in tobacco

***The method of designing vectors of transgenic codA:*** Restriction enzyme cleavage reactions, hybridization of recombinant vectors. The recombinant plasmid was transformed into bacterial cells by the pulse method of Cohen et al (1972).

***The method of transferring the designed vector structures into tobacco plants and checking the presence of transgenes***: according to the method of Topping (1998).

***The method of analyzing GB content in transgenic tobacco plants:*** *according to the method of Grieve et al. (1983).*

**2.3.2. Methods of studying suitable conditions for gene transfer in soybean variety DT22**

***The method of seed sterilization:*** Soybean seeds are sterilized with chlorine gas ($Cl_2$).

***The method of bacterial preparation:*** Types of media used in the process of culturing bacterial suspensions include: solid culture medium, liquid culture medium and bacterial suspension culture medium.

***The method of studying the influence of some factors on codA gene transfer efficiency:*** The influencing factors were tested at different thresholds and concentrations to select the optimal conditions for the gene transfer process.

**2.2.3. Methods of creating *codA* transgenic soybean plants**

***The method of genetic transformation and creation of transgenic soybean plants:*** Transferring genes into DT22 variety according to the method of Olhoft et al. (2001) with improvement. Transgenic soybean lines were screened with the herbicide PPT.

***The method of analysis of transgenic soybean plants:*** Total DNA was extracted according to the method of Delllaporta (1983). The presence of *codA* transgene in plants was confirmed by primer-specific PCR technique. The fusion of the *codA* transgene into the transgenic plant genome was checked by Southern blot technique. The expression of recombinant protein *codA* was checked by Western blot technique.

***The method of evaluation of drought tolerance of some transgenic plant lines under artificial drought conditions:*** The transgenic lines were evaluated for drought tolerance under artificial drought conditions set up in a growth chamber. Evaluation of GB indexes according to the method of Grieve et al (1983); MDA index, proline and POD activity according to the method of Chen and Zhang (2016).

**2.3.4. Data analysis and processing:** The data is processed by the software SPSS.

**2.3. RESEARCH LOCATION**

The experiment was carried out at the Institute of Biotechnology during the period from 2017 to 2020. The thesis was completed at the Department of

Genetics and Biotechnology, Department of Biology, University of Education, Thai Nguyen University.

## Chapter 3. RESEARCH RESULTS AND DISCUSSION

### 3.1. DESIGN AND EXPRESSION OF TRANSGENIC VECTOR CARRYING *codA* GENE

#### 3.1.1. Designing structures transferring *codA* gene

Three plant gene expression vectors *pBTII* carrying *35S, rd29A* and *HSP* promoters respectively to control *codA* gene expression in soybean were designed. After carrying out extraction and purification, the vectors *pIBTII* and *pCAMBIA-HSP-codA, pCAMBIA-35S-codA, pCAMBIA-rd29A-codA* were cut using two enzymes *hindIII* and *EcoRI*. The structure and size of the *pIBTII* vector and the *pCAMBIA* vector carrying the *codA* transgene in Figure 3.1 show that the plasmids have been completely cut into two bands with the theoretical size. The *pIBTII* vector is cut into two bands of 8.8 kb and 3 kb; vectors *pCAMBIA-35S-codA, pCAMBIA-rd29A-codA, pCAMBIA-HSP-codA* were cut into bands of about 11 kb and 2.4 kb, 2.7 kb and 2.6 kb in size corresponding to the size of *pCAMBIA* and transgenic structures *35S-codA, Rd29A-codA, HSP-codA.*

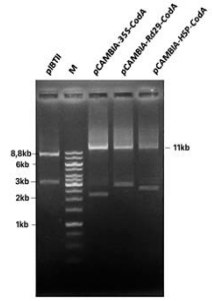


Figure 3.1. Electrophoresis of *pIBTII* and *pCAMBIA* vector products cut by *HindIII* and *EcoRI* enzymes

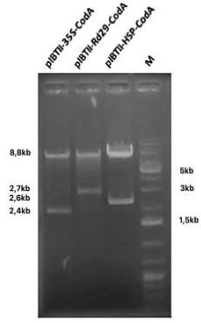


Figure 3.3. Electrophoresis of tested plasmid *pIBTII* carrying designed genes

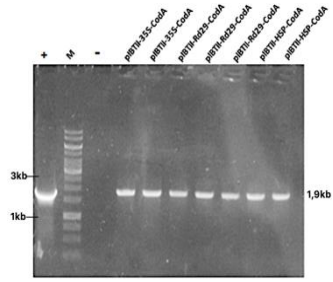


Figure 3.4. Electrophoresis of PCR products testing bacteria *A.tumefaciens* C58 carrying designed genes

Segments of transgenic vector *pIBTII* and segments of transgenic structures carrying *35S-codA, rd29A-codA, HSP-codA* were coupled together by T4 ligase reaction. The ligase product was transformed into E. coli DH5α transforming

bacterial cells by heat shock. Colony-PCR was tested with *codA-F/codA-R* specific primer pairs to select *E. coli* DH5α line transgenic vector carrying *codA*. Electrophoresis results showed that 7 tested colony lines were positive for colony-PCR, the obtained electrophoresis band was 1.9 kb in size, corresponding to the calculated *codA* transgenic vector size. Thus, the *codA* transgenic vector already exists in *E. coli* bacteria cells.

To further validate the accuracy of the newly designed vector, three positive colony lines containing *pIBTII-HSP-codA, pIBTII-35S-codA, pIBTII-rd29A-codA* vectors were randomly cultured and separated from plasmid. The plasmid was cut with *HindIII* and *EcoRI* enzymes. The products which underwent electrophoresis and photography shown in Figure 3.3 show that there were two bands appearing close to the 8.8 kb band of *pIBTII* and 2.4 kb, 2.7 kb and 2.6 kb of *pCAMBIA-35S- codA, pCAMBIA-rd29A-codA, pCAMBIA-HSP-codA*, which means that the tested colony lines carried the vector to be designed. All 7 positive colony lines were cultured in liquid LB medium supplemented with selective antibiotic (spectinomycine) to multiply the recombinant vector line for transformation into *A. tumefaciens* for gene transfer.

### 3.1.2. Evaluating the activity of vectors carrying the *codA* transgene in tobacco plants

*3.1.2.1. Creating transgenic tobacco plants through A. tumefaciens*

In order to test the activity of the *codA* transgenic vector, we transferred the *codA*-carrying structures into tobacco model plant of K326 variety and evaluated the selection efficiency of the Bar gene. The results of the transformation experiment showed that the percentage of shoots regenerated after gene transfer and selection was high in all three structures *pIBTII-HSP-codA, pIBTII-35S-codA, pIBTII-rd29A-codA*, reaching 88%, 90 %, 92% respectively and the shoots of 3 structures had survival rates on rooting medium supplemented with phosphinothricin of 42.64-54.23%. In contrast, the control leaf fragments had a shoot growth rate of only 27%, the shoots were small and died after 2 weeks of transformation. Transgenic tobacco lines that survived phosphinothrcin selection were grown using TN1 substrates under greenhouse conditions. After 30 days, young leaves were sampled, total DNA was extracted and checked for the

presence of *codA* transgene by PCR method with 2 pairs of primers *codA* F/R and bar F/R, the results are shown in Fig. 3.5.

The analysis results of Figure 3.5 showed that the electrophoresis band was about 750 bp and 400 bp in size, consistent with the size of the *codA* gene and the bar gene.

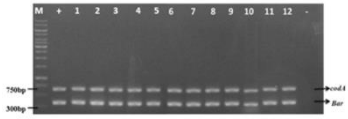


Figure 3.5. Electrophoresis of PCR product of transgenic tobacco lines

*M: marker 1 kb, well +: positive control, wells 1-5: transgenic lines rd29A-codA, wells 6 - 9: transgenic lines with 35S-codA structures, wells 10-12: transgenic lines with HSP-codA structures, well -: negative control.*

Figure 3.6. Selected transgenic tobacco shoots on medium supplemented with PPT 1.5 mg/l

*3.1.2.2. Results of evaluation and analysis of transgenic tobacco lines*

We kept inducing artificial drought *in vitro* for transgenic tobacco lines with PEG 8000 2.5%. The results showed that the transgenic tobacco plants in the artificial drought environment were able to maintain osmotic pressure and strongly develop their roots to enhance their resistance, the control plants in the artificial drought environment lost their osmotic imbalance, resulting in their underdevelopment and death.

We conducted an experiment of creating artificial drought for transgenic plants in a greenhouse. It was carried out in a growth chamber at 37°C, 55% humidity and absolutely without watering during the experiment. The results of phenotypic evaluation after 7 days of experiment showed that the transgenic tobacco lines still grew well while the control plants wilted after the 3rd day of drought and completely died after 7 days of drought. GB accumulation under drought conditions was also evaluated on day 7 of the experiment.

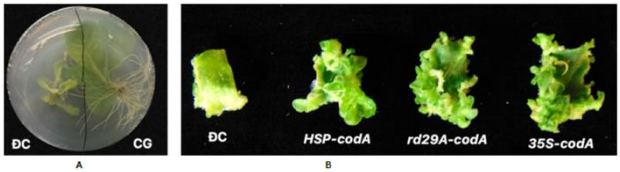


**Figure 3.7.** Results of physiological drought by PEG 8000 2.5% of transgenic tobacco lines

*A) shoots forming roots on physiological drought media;*

*B) Cotyledon fragments forming shoots on physiological drought media*

Quantitative results of cumulative GB under drought conditions in Figure 3.8 show that the transgenic plants had better drought tolerance than the control plants, specifically the average cumulative GB of *rd29A-codA, HSP-codA, 35S-codA* transgenic plants were 12.43 mg/g dry weight (mg/g KLT), 13.89 mg/g KLK, 12.89 mg/g KLK, respectively, compared with the cumulative GB content of control plant being 9.88 mg/g KLK. Thus, under drought conditions, the expression of *codA* gene improved the drought tolerance of transgenic plants compared with control plants by accumulating a large amount of GB. Thus, from the analysis results on tobacco plants, it can be concluded that the three transgenic vectors *pIBTII-HSP-codA, pIBTII-35S-codA, pIBTII-rd29A-codA* worked well in transgenic tobacco plants. and could be used as a material to transfer genes into soybean and other crops.

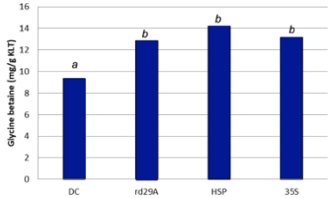


**Figure 3.8.** Quantitative results of GB content in transgenic tobacco

*Different letters in each column represent difference in P<0,05*

### 3.1.3. Discussing the results of designing vectors and the vector operation expressed on tobacco plants

Promoter is a factor that plays an important role in controlling gene expression. In our study, the *35S, HSP, rd29A* promoters were selected and became components of the structure carrying the *35S-codA, rd29A-codA, HSP-codA* transgene in the *pIBTII* transgenic vector. Under drought conditions, the high activity of these three vectors would help the *codA* transgene initiate transcription for the synthesis of choline oxidase, the key enzyme that catalyzes GB biosynthesis from choline in transgenic soybean, regulating intracellular osmotic pressure, reducing oxidative stress, thereby enhancing drought tolerance in *codA* transgenic soybean plants.

An efficient gene transformation process cannot be complete without efficient selection of a selective agent to differentiate between transgenic and

non-transgenic cells. The selective marker gene *bar* confers resistance to the herbicide PPT. The gene *bar* has been widely used and is an ideal marker for the identification of transgenic tissues under both greenhouse or field conditions. In addition, in order to detect and quantify recombinant protein in transgenic soybean by Western blot technique, the *codA* transgene-carrying structure is also attached with a DNA fragment encoding the cmyc antigen - a popular, economical and efficient choice of many current studies.

In our study, tobacco was used as a model plant to evaluate the activity of the expression vector carrying the *codA* transgene before it was transferred into soybean to improve drought tolerance. The result of *codA* gene expression in tobacco with a transgenic efficiency of 42.64-54.23% was a solid basis for us to continue to carry out *codA* gene expression in soybean and other crops.

### 3.2. IMPACTS OF SOME FACTORS ON THE EFFECT OF *codA* GENE TRANSFER IN SOYBEAN VARIETY DT22

#### 3.2.1. The impact of PPT concentration on the ability to produce soybean shoots


Figure 3.9. Cotyledon fragments on SIM2 selective medium with different concentrations of PPT after 14 days of culture

*A: 1 mg/l; B: 3 mg/l; C: 5 mg/l; D: 7 mg/l*

To determine the ppt content by selectively using experimental transgenic shoots, the shoot clusters were transferred to SIM2 medium, supplemented with ppt at 5 concentrations (0; 1; 3; 5; 7) mg/l. After two weeks of observation, it was found that after 14 days of culturing on SIM2 medium containing PPT 0 mg/l and 1 mg/l, the shoot clusters were green and developed normally, while the leaves of the shoot clusters turned yellow and fell off on day 7 on SIM2 medium containing 3-5 mg/l PPT. On SIM2 medium containing 7 mg/l ppt, the shoot clusters completely dried up after 8 days of culturing and the percentage of elongated shoots decreased with the increase of ppt concentration. After about 30

days, shoot clusters on the media supplemented with PPT concentration of 3 mg/l grew slowly, but there was the phenomenon of new and green shoot clusters being formed. At PPT concentration of 5 mg/l, shoots died completely. From the above analysis results, we decided to choose SIM medium supplemented with PPT 3.0 mg/l to select transgenic soybean plants.

**3.2.2. The impact of bacterial concentration, incubation time, and co-culture on the ability to induce soybean shoot formation**

*3.2.2.1. The impact of bacterial concentration on the induction of soybean shoot formation*

To determine the bacterial concentration of *Agrobacterium* at $OD_{650}$ used for gene transfer into soybean, we inoculated the soybean seed cotyledons damaged at the axils of the cotyledons with inoculum of different $OD_{650,}$ at 0,4; 0,6; 0,8 and 1,0 respectively during 30 minutes, then co-culture the infected cotyledons on CCM medium for 5 days. After 14 days, the cotyledons were transferred to SEM medium containing 1.5 mg/l PPT. The results in Table 3.3 show that, after 14 days of culture on SIM1 medium without PPT, there was not much difference in the ability to form shoots of cotyledon when infected with inoculum with the $OD_{650}$ values of 0,4 and 0.6. For cotyledons inoculated with inoculum with the $OD_{650}$ values of 0.8 and 1.0, the rate of re-infection of the cotyledons was very high, the cotyledons often died and did not produce multiple shoots. Cotyledon fragments infected with inoculum with the $OD_{650}$ values of 0.4 and 0.6 were further selected on SIM2 and SEM medium supplemented with PPT. After 14 days of selective culture, the cotyledon fragments inoculated with the $OD_{650}$ value of 0.6 continued to grow and form shoots, while the cotyledon fragments infected with the inoculum of the $OD_{650}$ value of 0.4 were almost completely dead on selective medium. Therefore, we selected the inoculum with the $OD_{650}$ value of 0.6 for transformation.

*3.2.2.2. The impact of incubation time of A. tumefaciens and co-culture on the induction of soybean shoot formation*

The study on the impact of incubation time of *Agrobacterium* and co-culture on the efficiency of gene transfer in soybean variety DT22 was carried out with incubation periods of 15 minutes, 30 minutes, 45 minutes respectively; co-culture

time of 2 days, 5 days and 8 days, the results presented in Table 3.4, show that, with the infection time of 15 minutes, the shoot clusters died almost completely at the last stage of the first selection; with the infection time of 45 minutes, there was a very high rate of re-infection and a lot of elimination at the SIM1 shoot induction stage. At 2-day co-culture, cotyledon fragments were much smaller than in 5-day and 8-day co-cultures, but at 8-day co-culture, the rate of re-infection was very high; on the $6^{th}$ day of bacterial co-culture, bacteria grew out of the filter paper and by day 8, some fragments were rotten (Figure 3.10). Therefore, infection time of 30 minutes and co-culture of 5 days gave the best results for transferring gene in soybean variety DT22.

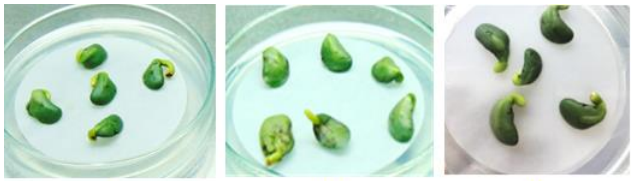


Figure 3.10. Cotyledon fragments after different co-culture times

*A: 2 days; B: 5 days; C: 8 days*

**3.2.3. The impact of antibiotic concentration and bacterial cleaning time on the formation and growth of soybean shoots**

Determination of antibiotics and bactericidal time after 5 days of co-culture is an important factor on the ability to kill bacteria and form multiple shoots of cotyledons after infection with recombinant *A. tumeffaciens*. Using broad-spectrum bactericidal antibiotic cefotaxime with concentrations of 200 mg/l, 500 mg/l, and 1000 mg/l, respectively to supplement the bacterial cleaning medium (liquid SIM) with a wash time of 5 minutes, 10 minutes, and 15 minutes. The results through each stage showed that supplementing the cefotaxime concentration of 500 mg/l to the bacterial cleaning medium for 10 minutes was the best. At the cefotaxime concentration of 1000 mg/l, the cotyledon fragments formed multiple shoots poorly, the damaged axils of the cotyledons became black and did not form multiple shoot clusters. At the cefotaxime concentration

of 200 mg/l, cotyledon fragments were highly re-infected. After only the first week of shoot induction, most of the cotyledons were re-infected with bacteria.

### 3.2.4. Discussing the results of the study on the impacts of some factors on the effect of *codA* gene transfer in soybean variety DT22

Currently, many gene transfer procedures have been applied to soybean, but the most common one is transgenic transformation by infecting recombinant *A. tumefaciens* through the axillary cotyledons. However, this method still has some limitations that need to be overcome and the effect of gene transfer depends on the genotype of each soybean variety. Therefore, the study of selecting conditions for gene transfer which is appropriate to the genotype of each variety has been noticed and interested by researchers on transgenic soybean. The results of our study have shown that the impacts of some factors affecting the effect of *codA* gene transfer into the Vietnamese soybean variety DT22 have been surveyed and evaluated, including phosphinothricin concentration, bacterial concentration, incubation time of *A. tumefaciens*, co-culture, selective antibiotic concentration and bacterial cleaning time. Efficient selection of a selective agent is an important step in a gene transformation process. The results of our study showed that using PPT 3 mg/l at the shoot induction stage in SIM medium and PPT 1.5 mg/l at the shoot elongation stage in SEM medium gave the most effective selection. Besides, bacterial concentration upon infection, *Agrobacterium* incubation and co-culture is a factor that greatly affects the effect of gene transfer through *A. tumefaciens*. In the results of our study, the soybean cotyledonous fragments that were injured at the axillary of the cotyledons were infected with bacterial solution with different $OD_{650}$ values, the bacterial solution with $OD_{650}$ value = 0.6 with 30-minute incubation, 5 day co-culture in the dark are suitable for shoot induction and shoot elongation on selective media. In addition, the determination of antibiotics and the bacterial cleaning time after 5 days of co-culture were important factors for the bactericidal ability and multiple shoot formation of cotyledons after infection with recombinant *A. tumeffaciens*. The results through each stage showed that the cefotaxime concentration of 500 mg/l supplemented to the bacterial cleaning medium for 10 minutes gave the best results for gene transfer in soybean variety DT22.

Thus, from the results of evaluating the impacts of factors on transformation and multiple shoot formation in soybean variety DT22, we have identified factors suitable for *codA* gene transfer and multiple shoot formation in soybean variety DT22. This result is the premise for us to complete the *codA* gene transfer process into the soybean variety DT22 to serve the next research of the topic.

### 3.3.1. Results of *codA* gene transfer into soybean variety DT22

The results of transforming transgenic vector into soybean cotyledons described in Table 3.6 showed that the number of transformed samples for each structure was about 3000, in which the number of samples forming multiple shoots reached the rate from 62.75% to 73.55%. From these shoot clusters, after 3 stages of shoot elongation, 128, 138, and 140 elongated shoots were obtained with *HSP-codA, 35S-codA, and rd29A-codA* structures, respectively. The shoots reached a height of 2.5–3.5 cm and had the main leaves cut to rooting medium. Rooting shoots that produced complete plants were grown on TN1 substrate supplemented with 25% tribat. After acclimatization process in a growth chamber at 28$^{o}$C, 80% humidity, light/dark cycle of 16/8, a total of 60 soybean plants of three transgenic structures grew and developed well.

### 3.3.2. Analysis of transgenic soybean plants

*3.3.2.1. Screening transgenic plants with herbicides and PCR*

PPT 250mg/l was spread directly on the leaf surface of soybean lines $T_0$. After 3 days of herbicide application, the leaves at the spread area of the control plants and some transgenic lines shrank, gradually lost the green pigment of the leaves and turned yellow; after 5 days of spreading, the spread area of the leaves turned yellow-brown and became dry. In addition, some transgenic lines did not show signs of herbicide damage at the tested site, and the leaves did not lose their color. Thus, after screening with herbicides, we obtained 29 out of 60 lines of transgenic $T_0$ soybeans which were not susceptible to ppt 250mg/l. The $T_0$ lines were tested by PCR with 2 pairs of primers *codA F/R* and *Bar F/R*. As a result, we obtained 27 lines with positive results, including 8 lines of *HSP-codA*, 10 lines of *35S-codA* and 9 lines of *rd29A-codA* (Figure 3.14).

From 27 $T_0$ lines obtained, we collected their seeds and sew to the $T_1$ generation, screening and testing by PPT 250mg/l, 8 lines which were not susceptible to ppt included 6 *rd29A-codA* lines and 2 *HSP-codA* lines. After these 8 lines were PCRed with 2 pairs of specific primers, obtained 4 lines of *rd29A-codA* (D2, D3, D4, D7) and 2 lines of *HSP-codA* (H3, H11), we obtained two bands with sizes of approximately 750bp and 400bp suitable with the size of the corresponding *codA* and *bar* gene fragments shown in Figure 3.15.

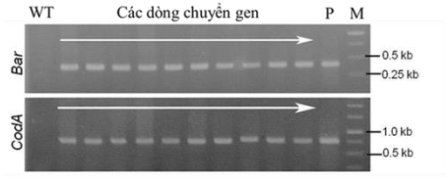


Figure 3.14. Results of testing $T_0$ by PCR

*M: standard DNA scale 1kb; P: A. tumefaciens carrying recombinant vectors;*

*WT: soybean leaf sample DT22*

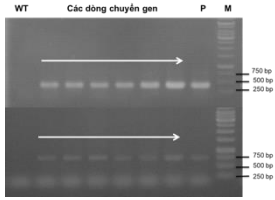


Figure 3.15. Results of testing $T_1$ by PCR

*M: standard DNA scale 1kb; P: A. tumefaciens carrying recombinant vectors;*

*WT: soybean leaf sample DT22*

The above results show that the number of transgenic lines decreased in the $T_1$ generation due to the small number of seeds obtained from *in vitro* $T_0$ plants, and the uneven seed quality affecting the germination of seeds in the next generation. Thus, we obtained 27 lines of soybean carrying *codA* gene in the $T_0$ generation and 6 lines in the $T_1$ generation, the gene transfer efficiency is about 0.33% in the $T_0$ generation. To test transgenic soybean lines in the next generations, we used four $T_1$ lines (D2, D3, D4, D7) carrying the drought-tolerant inducible promoter *rd29A* for the active expression of this promoter, two $T_1$ lines (H3, H11) carrying the *codA* gene under the activity of the heat-inducible promoter *HSP* were seeded and preserved with silica gel.

*3.3.2.2. Testing codA transgene fusion and expression in transgenic soybean lines by Southern blot and Western blot techniques*

Four $T_1$-generation *rd29A-codA* transgenic soybean lines were tested by Southern blot technique to demonstrate the fusion of the *codA* transgene into the transgenic soybean genome and determine the number of copies of the transgene.

Southern blot analysis results in Figure 3.16 showed that DNA hybrid bands appeared in all four transgenic soybean lines tested. Lines D3, D4 and D7 had 1 hybrid band; and line D2 had 2 hybrid bands. This indicates that the *codA* gene had 1 copy in the D3, D4 and D7 lines, and 2 copies in the D2 line. Hybrid bands of two lines D3 and D4 implied that these two lines may have been generated from the same transgenic group. The results of this analysis demonstrated the fusion of the *codA* transgene into the transgenic soybean genome.

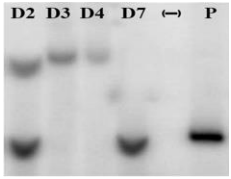


Figure 3.16. Results of analysis by Southern blot on transgenic soybean lines of $T_1$ generation

*D2-D7: transgenic soybean lines $T_1$ generation with structures rd29A-codA; (-): negative control; P: Plassmid pIBTII-35S-codA as positive control*

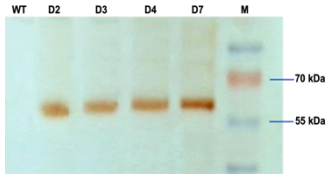


Figure 3.17**.** Results of analysis by Western blot on transgenic soybean lines of $T_1$ generation

*D2-D7: transgenic soybean lines $T_1$ generation with structures rd29A-codA; M: Marker; WT: non-transgenic soybean plants*

Next, we continued to analyze the expression of *codA* transgene on 4 $T_1$-generation transgenic lines (D2, D3, D4, D7) through determining the presence of recombinant protein by Western blot. The recombinant protein of the transgenic structure *codA* contained the cmyc antigen at the C end, so this recombinant protein could be detected by Western blot. When we conducted Western blot technique on young leaf samples of $T_1$ generation *rd29A-codA* transgenic soybean lines to evaluate the expression of transgenes in plants, we obtained the results shown in Figure 3.17. The results of Western blot analysis showed that the protein bands ~60 kDa in size were equivalent to the choline oxidase molecular weight encoded by the *codA* gene when looked up on NCBI bank. In Figure 3.17, all transgenic plants had clear bright bands, while WT plants had no equivalent bands. The results of recombinant choline oxidase protein expression analysis in *codA* transgenic soybean showed that the transgene carrying the structures *rd29A-codA* and *HSP-*

*codA* was inherited from the $T_0$ generation to the $T_1$ generation and was expressed as recombinant protein choline oxidase.

### 3.3.3. Evaluation of drought tolerance of some transgenic soybean lines

*3.3.3.1. Evaluation of seed germination under artificial drought conditions*

The seed germination and stem formation stages are critical for plant growth and development that are particularly sensitive to water-deficient conditions. Therefore, the seeds of the transgenic soybean lines in the $T_1$ generation were evaluated under artificial drought conditions by adding 10% or 15% PEG 8000 to the nutrient medium. After 5 days of culture on medium without PEG 8000 (0% PEG 8000), no difference in stem length was observed between the DT22 line and the transgenic lines shown in Figure 3.18. This showed that all the seeds of the experimental lines germinated well and had a stem length of approximately 5 cm in the GM medium without PEG 8000. In contrast, on the medium supplemented with 10% PEG8000, the seeds of the transgenic lines and DT22 germinated; however, the length of the sprouts of the soybean lines was different. The non-transgenic DT22 line showed a decrease in the stem length to less than 4 cm, much shorter than that of the transgenic lines, while the seeds of the transgenic lines maintained normal growth. On medium containing 15% PEG 8000, the germination and growth of both DT22 and transgenic lines were significantly reduced. Seed growth on medium was inhibited. However, the seeds of the transgenic line still had a higher growth rate and a significantly greater stem length than that of the DT22 line shown in Figure 3.19. Thus, thanks to the expression of *codA* gene, soybean seeds had better germination and growth than non-transgenic lines (DT22) under *in vitro* artificial drought conditions.

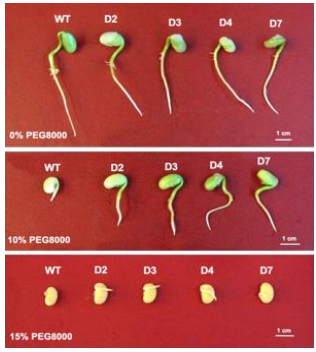


**Figure 3.18.** Germination ability of soybean after 5 days of culture in artificial drought medium
*D2-D7: transgenic soybean lines $T_1$ generation with structures rd29A-codA; WT: non-transgenic soybean plants of DT22*

*3.3.3.2. Evaluation of the growth ability and drought tolerance of transgenic soybean plants under artificial drought conditions*

To evaluate the growth and development of soybean under artificial drought conditions, the seeds of the *codA* transgenic soybean lines and the non-transgenic DT22 soybean lines were grown individually in different pots and examined by spreading herbicide. In the second phase of the experiment, soybean plants were treated with artificial drought in the growth chamber. During the experiment, the transgenic and DT22 soybean plants placed under control conditions grew and developed normally under adequate watering conditions, and no difference in plant morphology between the plant lines was noticed.

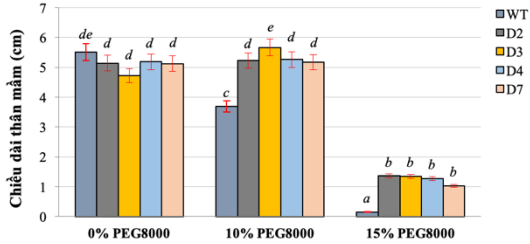


Figure 3.19. Length of stem of soybean lines in artificial drought medium

*D2-D7: transgenic soybean lines $T_1$ generation; WT: non-transgenic soybean plants of DT22; Different letters in each column represent difference in P<0,05*

In contrast, under artificial drought conditions, the growth rate of the soybean line DT22 showed signs of slowing down compared with that of the transgenic lines. Moreover, on the 9th day of experiment, under drought conditions, the leaves of the soybean line DT22 showed signs of yellowing, drying and wilting. Meanwhile, despite showing signs of growth loss such as wilting, the soybean leaves of the transgenic lines were still able to recover after being watered again (Figure 3.20). The average fresh weight of the experimental soybean lines was also determined to evaluate the growth rate of the transgenic soybean plants under artificial drought conditions. The biomass obtained on day 9 of soybean lines in the artificial drought tolerance experiment is shown in Figure 3.21.

The results of the chart analysis showed that, under control conditions, the biomass of the soybean lines (DT22 line and transgenic lines) was similar, which coincided with the results of growth evaluation of the soybean lines through outside observation. In contrast, there was a significant difference in the biomass obtained from the transgenic lines and the DT22 lines under drought conditions. Specifically, for the line DT22, the biomass obtained under drought conditions was only half (reaching 2.56 g) of what obtained under the control condition (reaching 6.32 g). Fresh weight and dry weight/fresh weight ratio of the experimental soybean lines on the 9th day of experiment in both control and drought conditions showed that the transgenic lines also had a decrease in biomass in drought condition compared with that of control one, but significantly higher than that of DT22 line. Thus, the *codA* transgenic soybean lines maintained higher growth rates and biomass than non-transgenic soybean plants under artificial drought conditions..

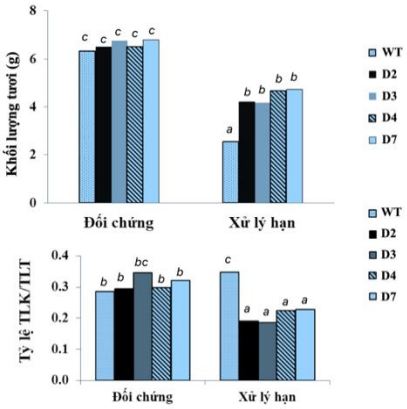


Figure 3.21. Biomass obtained on day 9 of soybean lines in artificial drought tolerance experiment

*Different letters in each column represent difference in P<0,05*

*3.3.3.3. Evaluation of biochemical indexes of transgenic soybean plants under artificial drought conditions*

Analysis of GB content in Figure 3.22 showed that, in all transgenic soybean leaves, GB content was higher than that in non-transgenic DT22 leaves. Specifically, the GB content ranged from 34.1 to 39.5 µg/mg dry weight (DW). The D2 soybean line had the highest cumulative GB content of 39.5 µg/mg DW and the D4 line had the lowest cumulative GB content of 34.1 µg/mg DW, while in the leaves of the non-transgenic soybean line DT22, the GB content only reached 13.7 µg/mg DW. The proline content of the non-transgenic line DT22

was only 102.9 µg/g fresh weight (FW), while it was high in all four transgenic soybean lines and increased to 219.6 µg/g FW in line D4 (Figure 3.23).

The MDA cell membrane damage index in Figure 3.24 showed that the MDA content in all *codA* transgenic soybean lines was clearly lower than that of the control lines. Under drought conditions, the MDA content of wild-type leaves was high, at 1.234 nmol/g FW, while the MDA content in transgenic soybean decreased from 0.702 to 0.454 nmol/g FW. These results indicate that *codA* transgenic soybean plants exhibit less severe oxidative stress and less damage than non-transgenic plants. POD activity is also known as an indicator of the tolerance of soybean cultivars against water deprivation. Analysis of POD activity in Figure 3.25 showed that in all transgenic soybean lines, it was significantly higher than that of non-transgenic soybean line DT22 (with only 2.08 U/g FW), in which transgenic soybean line D2 showed the highest POD activity (reaching 8.4 U/g FW). This result is consistent with the MDA data in Figure 3.24, where the lowest MDA content was observed in the D2 transgenic line (0.45 nmol/g FW).

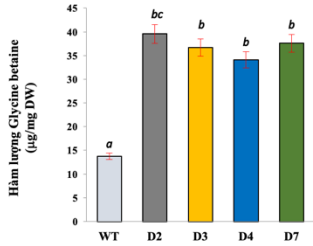


Figure 3.22. GB content of drought tolerant soybean lines

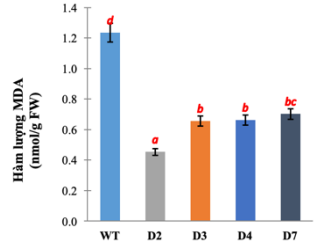


Figure 3.24. MDA content of drought tolerant soybean lines

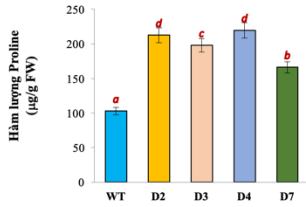


Figure 3.23. Proline content of drought tolerant soybean lines

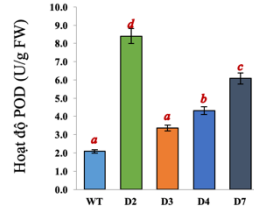


Figure 3.25. POD activity of drought tolerant soybean lines

*D2-D7: transgenic soybean lines T1 generation; WT: non-transgenic soybean plants of DT22*
*Different letters in each column represent difference in P<0,05*

### 3.3.4. Discussion of results of *codA* gene transformation into DT22 soybean variety and testing for drought tolerance of some transgenic soybean lines

In this study, *codA* gene from bacteria *A. globiformis* which was artificially synthesized to optimize gene expression in plants under the control of promoters *rd29A, HSP* and *35S* was successfully transformed into soybean plant. The analysis results of transgenic plants by PCR and Southern blot techniques confirmed the presence of *codA* in transgenic soybean plants. The transgenic soybean plants showed faster growth and greater biomass production than the non-transgenic control lines in artificial drought conditions. In addition, the accumulation of GB, proline and POD activity also increased in transgenic soybean under *codA* gene expression in transgenic soybean. This result is consistent with several recently published studies on the enhancement of *codA* gene expression through the accumulation of GB content, proline and POD activity in some transgenic plants.

In this study, the GB content accumulated in *codA* transgenic soybean was higher than that in the non-transgenic soybean line under drought stress. GB accumulated at a higher level can enhance osmotic pressure, reduce water loss due to drought stress in transgenic soybean, thereby maintaining high vegetative growth rate and biomass production than non-transgenic plants. In addition to GB, free proline is also accumulated in plants under environmental stress. After 9 days of drought treatment, the proline content in *codA* transgenic soybean plants increased 1.5-2 times compared with non-transgenic DT22 soybean plants. Our study contributes to confirming the importance of *codA* gene in drought tolerance of soybean.

Increased levels of MDA, a peroxidative-producing unsaturated fatty acid, are an indicator of cell membrane damage. In addition, the activity of POD is also an important indicator in assessing the tolerance of soybean genotypes under water shortage conditions. The increase in POD activity was associated with higher drought tolerance of the transgenic soybean varieties. *CodA* transgenic soybean plants had half the MDA content and fourfold POD activity than non-transgenic soybean plants under drought conditions, indicating the ability to reduce oxidation and limit membrane-damaging effects of *codA* transgenic soybean plants. The large changes in MDA and POD concentrations could be attributed to the role of transgene *codA* and promoter *rd29A* in transgenic soybean.

# CONCLUSIONS AND RECOMMENDATIONS

## Conclusion

1. The three transgenic vectors *pIBTII-35S-codA, pIBTII-rd29A-codA,* and *pIBTII-HSP-codA* carry the *codA* gene encoding choline oxidase, under the control of three different inducible promoters: *35S, rd29A* and *HSP* were successfully designed and transformed into tobacco. Under adverse conditions of temperature, humidity and water, transgenic tobacco lines survived and developed by accumulating higher levels of glycine betaine than non-transgenic tobacco plants.

2. The factors suitable for transferring *codA* gene in the *pIBTII/rd29A-codA* structure into the soybean variety DT22 were identified. The concentration of PPT 3 mg/l at shoot induction stage in SIM medium and the concentration of PPT 1.5 mg/l at shoot elongation stage in SEM medium gave the highest selective efficiency. *A. tumefaciens* solution with the value of $OD_{650}$=0.6 with incubation time of 30 minutes, co-cultured for 5 days in the dark and killed bacteria with cefotaxime 500 mg/l are suitable for shoot induction and shoot elongation on selective medium.

3. The structure *pIBTII/rd29A-codA* was successfully transformed into the soybean variety DT22 and created 4 *codA* transgenic soybean lines in the $T_1$ generation. Transgenic soybean lines in the $T_1$ generation which were tested by Southern blot and Western blot, and evaluated for drought tolerance in artificial conditions could grow well under stress conditions. Expression of the transgene *codA* under the control of the *rd29A* promoter increased the transcriptional activity of the *codA* gene and increased biomass in transgenic plants. The GB content increased significantly in the *codA* transgenic soybean lines, ranging from 34.1 to 39.5 µg/mg dry weight, an increase of 248.9% - 288.3% compared to that of the non-transgenic plants. Proline content increased 1.5-2 times, POD activity increased 4 times compared with that of non-transgenic plants and MDA content decreased 0.5 times under drought conditions.

## Recommendations

It is necessary for the transgenic soybean lines to be continuously monitored and evaluated on the stability of the transgene across generations and applied for further studies.

## PUBLICATIONS RELATED TO THE DISSERTATION

1. Dong Thi Ta, Dung Manh Ngo, Nhung Hong Nguyen, Ngoc Bich Pham, Phat Tien Do, Ha Hoang Chu (2020), "Production of drought tolerant transgenic soybean expressing *codA* gene under regulation of a water stress inducible promoter", *Pakistan Journal of Botany*, 52(3), pp. 793-799, (SCIE).

2. Manh Dung Ngo, Dong Thi Ta, Ngoc Bich Pham, Hoang Ha Chu, Hoang Mau Chu (2020), "The influence of some factors to the efficiency of coda gene transformation into the DT22 soybean variety", *TNU Journal of Science and Technology*, 225(11), pp. 121-127.

3. Ngo Manh Dung, Ta Thi Dong, Nguyen Hong Nhung, Nguyen Van Doai, Chu Hoang Ha, Chu Hoang Mau, "Structure and activity of plant transgenic vector carrying gene encoding choline oxidase", *TNU Journal of Science and Technology*, 226(14), pp. 297-304.