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Establishment of transgenic potato cultivar IPB CP1 plants containing gene encoding for superoxide dismutase to increase the abiotic stress tolerance

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ABSTRACT Potato (*Solanum tuberosum* L.) cultivar IPB CP1 is suitable as a raw material for the potato chip industry. Potato plants are sensitive to various abiotic stresses such as drought, aluminium and salinity, which induce reactive oxygen species (ROS). ROS is very toxic to plant cells. Superoxide dismutase (SOD) is one of the enzymes that catalyse ROS to H_2O_2 and O_2. This study aimed to establish transgenic potato cv. IPB CP1 plants containing the *MmCuZn-SOD* gene that are tolerant to various abiotic stresses. Genetic transformation using internodes without buds as explants produced putative transgenic potato with a transformation efficiency of 51.25% and a regeneration efficiency of 38.87%. Integration analysis of the *MmCuZn-SOD* transgene in putative transgenic plants by polymerase chain reaction (PCR) with a set of specific primers showed that eight plants contained the *MmCuZn-SOD* gene under the control of the 35S CaMV promoter. In vitro salinity stress, aluminium stress, and drought stress assays showed that transgenic plants had a higher number of roots and total root length than non-transgenic ones. These results indicate that transgenic potato cv. IPB CP1 plants are more tolerant to abiotic stresses than non-transgenic ones.

KEYWORDS abiotic stress tolerance; *MmCu/Zn-SOD* gene; potato cv. IPB CP1; transgenic

1. Introduction

Potato is the fourth most consumed staple food worldwide after rice, wheat, and corn (Zhang et al. 2017). As a national commodity in Indonesia, the potato receives significant attention from the government for its development. Potato production in Indonesia in 2019 was 1.3 million tons (FAOSTAT 2021), an increase 29,000 tons from the previous year. However, the issue of growing potatoes is sensitive to various abiotic stresses. Planting potatoes in fields conversed from tea plantations is hampered by soil acidity and high Al solubility. In addition, climate change and rainfall distribution patterns cause drought in potato fields that do not have an irrigation system. These abiotic stresses can reduce potato production. One effort to deal with this problem is to use cultivars tolerant to abiotic stress. Introducing the gene encoding for superoxide dismutase into the plant can establish drought and aluminium-tolerant plants.

Abiotic stresses such as drought, low or high temperature, high solubility of aluminium, and high salinity can trigger the production of Reactive Oxygen Species (ROS). ROS can cause cell damage due to protein oxidation, enzyme inactivation, changes in gene expression, DNA damage, and membrane decomposition (Gill et al. 2010; Sharma et al. 2012; Jajic et al. 2015). The SOD enzyme is one of the antioxidants that can catalyse superoxide radicals into O_2 and H_2O_2 molecules so that plants can tolerate various abiotic stresses (Bowler et al. 1992). The expression of SOD can be increased when the plants grow under the condition of various abiotic stresses such as drought (Wang et al. 2005), aluminium (Du et al. 2010), low temperature or cold (Xu et al. 2014), and salinity (Jing et al. 2015), or under biotic stresses (Tertivanidis et al. 2004; Lu et al. 2017).

MmCu/Zn-SOD gene was isolated from *Melastoma malabathricum* L. and constructed into pGWB-MmCu/Zn-SOD binary plasmid expression vector under the control of the strong 35S CaMV promoter (Hannum 2012). Potato cultivar IPB CP1 is very suitable to be used as raw material for producing potato chips. However, IPB CP1, like other potato varieties, is not tolerant to drought stress, aluminium, and salinity. Therefore, this study aimed to establish transgenic potato cv IPB CP1 plants containing the *MmCu/Zn-SOD* gene under the control of 35S CaMV strong promoter. The transgenic potato

cv IPB CP1 plants were expected to be tolerant to salinity, aluminium, and drought stress.

2. Materials and Methods

2.1. Multiplication of plant materials and Agrobacterium tumefaciens

A single node of stem containing one bud of potato cv IPB CP1 plant was cultured in vitro on MS media (Murashige and Skoog 1962), in the culture room at 24-25 °C with a photoperiod of 16 h and lighting 2,000-3,000 lux, for four weeks.

Agrobacterium tumefaciens containing the pGWB5-MmCu/Zn-SOD plasmid were cultured in liquid LB medium supplemented with antibiotics 50 mg/L hygromycin, 50 mg/L kanamycin, and 50 mg/L streptomycin. Bacterial cultures were placed on the shaker at 150 rpm in the dark at room temperature for 8-12 h to reach an optical density at 600 nm (OD_{600}) around 0.3-0.5. The map of the T-DNA region in the pGWB5-MmCu/Zn-SOD plasmid is presented in Figure 1.

2.2. Genetic transformation of potato plant

Internode without buds of 1 cm long was cultured on MS containing 2 mg/L 2.4-D and 0.8 mg/L zeatin as preculture (PC) media and stored in the culture room for one day. The pre-cultured explants were then soaked in *A. tumefaciens* culture in a liquid co-cultivation medium, that was MS medium containing 2 mg/L 2,4-D, 0.8 mg/L Zeatin, and 20 mg/L acetosyringone, pH 5.8 for 10 mins. The explants were dried using aseptic tissue for 10 mins and then cultured on solid co-cultivation medium and stored in the dark for three days. The explants were then washed with sterile distilled water two times and then soaked in a solution of cefotaxime 200 mg/L for 10 mins while shaking and then dried with aseptic tissue for 10 mins. The explants were grown on a callus induction medium that was MS medium containing 20 g/L glucose, 3 mg/L zeatin, 1 mg/L IAA, 0.5 mg/L GA3, and 2.5 g/L agar Gelzan™ during 14 days without a selection agent. Then, the calli were transferred to a selective medium that is a callus induction medium containing 10 mg/L hygromycin. Putative transgenic shoots growing from callus in the selective medium were grown on MS medium containing 10 mg/L hygromycin. The plantlets were sub-cultured every four weeks. Hygromycin concentration in each subculture was then increased to 20 and 30 mg mg/L. The number of developed calli, and hygromycin-resistant calli, were counted to determine the transformation and regeneration efficiency.

2.3. Molecular analysis of putative transgenic plants

The total DNA of potato plants was isolated as described by Suharsono (2002), which was modified using CTAB 2X buffer. The quantity of DNA was measured using a UV spectrophotometer (NanoVue Plus™, Biochrom, UK). DNA integrity was determined based on the integrity of the actin gene, which was amplified by PCR using primer pair of Act-F (5'-ATG GCA GAT GCC GAG GAT AT-3') and Act-R (5'-CAG TTG TGC GAC CAC TTG CA-3'). The analysis of the integration of the *MmCu/Zn-SOD* gene in the plant genome was carried out by PCR using a specific primer pair, i.e., 35SF: 5'-AAA CCT CCT CGG ATT CCA TT-3' and SODR2: 5 '-CAT CTC CAA CGG TGA CAT TG-3' (Hannum 2012). The PCR was carried out under the condition of pre-PCR at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 40 s, annealing at 58 °C for 45 s, and extension at 72 °C for 1 min, and terminated by post-PCR at 72 °C for 10 mins and the PCR temperature then lowered to 25 °C for 10 mins. The PCR results were then electrophoresed in 1% agarose gel with a voltage of 100 volts for 28 mins in TAE buffer solution. The gel was soaked in 0.5 mg/L ethidium bromide for 10 mins and rinsed with water for 5 mins. The electrophoresis products were visualized using a UV transilluminator.

2.4. Drought, aluminium and aalinity tolerance assay

An assay of the abiotic tolerance of potato plants to drought, aluminium, and salinity stresses was done separately. This experiment was carried out by using a completely randomised design with one treatment and three replications, in which each replication consisted of four plantlets. The stem fragments containing single buds were cultivated in the bottle containing liquid MS medium and filter paper to support the explants, supplemented by a stress treatment solution. The treatment solution for drought stress was 10% PEG 8000, aluminium stress treat-

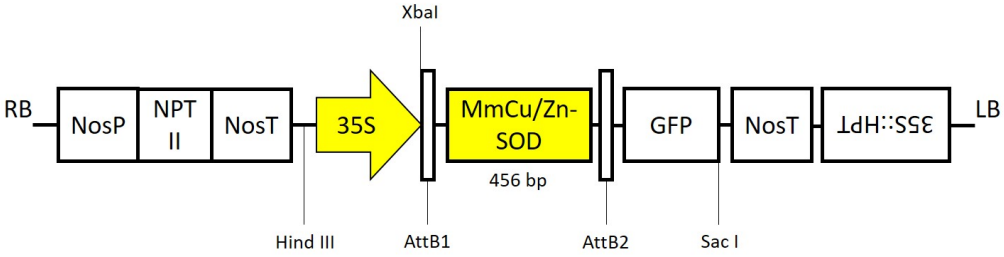


FIGURE 1 Map of the T-DNA region of the pGWB5-MmCuZn-SOD plasmid. RB: right border, NosP: nopalin synthase promoter, NosT: Nos terminator, NPTTII: neomycin phosphotransferase II, 35S: 35S CaMV promoter, MmCu/Zn–SOD: superoxide dismutase, HPT: hygromycin phosphotransferase, AttB1 and AttB2 = Getway system recombination sites (Hannum 2012).

ment used 2 mM $AlCl_3.6H_2O$ at pH 4.0, and salinity stress treatment used 125 mM NaCl. The explants grown in MS medium without stress treatment solution were used as controls. The total number of roots and total root length was observed three weeks after cultivation to determine the abiotic stress tolerance.

3. Results and Discussion

3.1. Genetic transformation of potato cultivar IPB CP1 plant

The *MmCu/Zn-SOD* gene linked to *hpt* gene has been successfully introduced into potato IPB CP1 indicated by resistant plants to hygromycin. Stem fragments were used as explants in this experiment because they were more effective than leaf explants for bud regeneration (Visser et al. 1989). *Agrobacterium tumafaciens* is widely used as an intermediary to introduce genes into plants to produce transgenic plants. The T-DNA region in the plasmid contained by *A. tumefaciens* is efficiently transferred into the host cells and the genes in the T-DNA region can be expressed in the host cells (Gelvin 2017).

The explants started to swell slightly in the pre-culture medium one day after cultivation (Figure 2a). Pre-culture before *A. tumefaciens* inoculation aimed to increase the transformation efficiency. In tomatoes, pre-culture leaf cotyledon explants for six days increased the transformation efficiency (Rai et al. 2012). All explants grown in a callus-induction medium without hygromycin formed calli 14 days after planting (Figure 2b). Cefotaxime was used to kill *A. tumefaciens* rapidly growing on the calli. The use of cefotaxime and timentin in a callus-formation medium was able to inhibit the growth of Agrobacterium (Kumar et al. 2017). After being sub-cultured in a selection medium containing 10 mg/L hygromycin, some calli continued to grow, and others died. Calli sensitive to hygromycin became browning, while others being resistant were green. Resistant calli to hygromycin regenerated to form putative transgenic shoots four weeks after planting (Figure 2c). Zeatin was added to the selection and regeneration medium to increase shoot regeneration. The addition of zeatin to the shoot culture of strawberry stems increased the number of regenerated shoots (Haddadi et al. 2013). The regenerated shoots from the calli were cut and planted separately to induce putative transgenic shoots to form roots and multiply the plants (Figure 2d).

Genetic transformation to 240 explants of potato cv. CP1 with the *MmCuZn-SOD* gene resulted in 123 resistant calli to 10 mg/L hygromycin, so the transformation efficiency in this study was 51.25%. From 123 resistant calli, 45 calli regenerated resistant shoots to hygromycin, so the regeneration efficiency of the transgenic shoot was 38.87% (Table 1). This transformation efficiency was higher than that obtained by Gea et al. (2017), who carried out the genetic transformation of potato cultivar CP1 with the *Hd3a* gene, and Pasmawati et al. (2021), who carried out the genetic transformation of potato cultivar IPB CP3 with the *LYZ-C* gene. The difference in transformation and regeneration efficiency of genetic transformation of plants

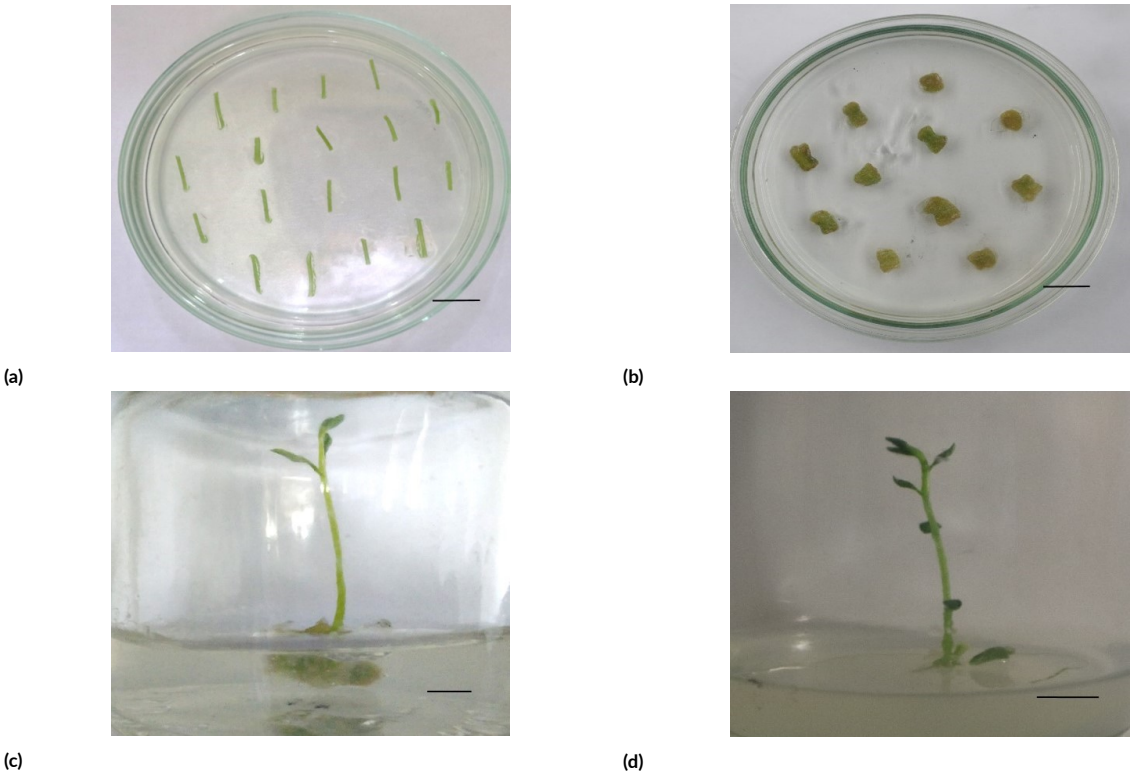


FIGURE 2 Stages of establishment of transgenic potato cv. IPB CP1 carrying *MmCuZn-SOD* gene. (a) Explant of internode in pre-culture medium, (b) Callus formation, (c) Shoot regeneration, (d) Putative transgenic plant. Bar= 1 cm.

TABLE 1 The transformation and regeneration efficiency of transgenic potato cv. IPB CP1.

Experiment	Total explant	Total Resistant Callus	Total Regenerated Callus	Transformation Efficiency (%)	Regeneration efficiency (%)
1	80	35	22	43.75	62.86
2	80	48	9	60	18.75
3	80	40	14	50	35
Total	240	123	45	-	-
Averages	80	41	15	51.25	36.59

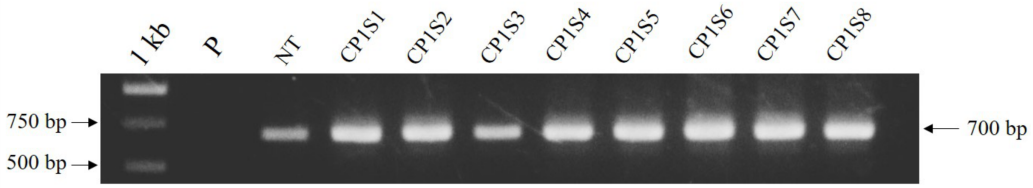
mediated by *A. tumefaciens* is caused by several factors such as pre-culture conditions of explants, co-cultivation period, the composition of the medium, mainly containing auxin, post-co-cultivation treatment, and concentration of antibiotics used (Cervera et al. 1998).

Each resistant callus to hygromycin produced one shoot, so this study resulted in 45 independent putative transgenic shoots. The shoots were sub-cultured in a selection medium containing hygromycin in which its concentration gradually increased, up to 30 mg/L hygromycin. Eight out of the 45 transgenic putative, showing the fast-growing, were selected to be multiplied and further analysed. Each of them was named a clone.

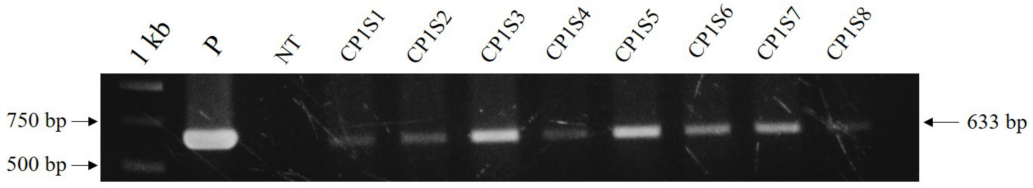
3.2. Molecular analysis of transgenic potato

PCR was performed to analyse the integration of *MmCu/Zn-SOD* gene into putative transgenic plants. DNA of potato plant consisting of eight putative transgenic clones and one non-transgenic clone was successfully isolated. The success of DNA isolation and the integrity of genomic DNA were analysed based on the actin gene. PCR to actin gene showed that eight clones of putative transgenic plants and non-transgenic plants contained about 700 bp of actin genes, while the pGWB5-MmCu/Zn-SOD plasmid did not contain actin genes (Figure 3a). This result indicates that DNA isolation has been successful and genomic DNA from transgenic and non-transgenic plant clones had high integrity. Actin is the housekeeping gene, abundant and present in all cells. Actin is usually used as the internal control for gene expression analysis, although the level of actin gene expression in all cell types from chickens is not always high (Lin and Redies 2012).

The integration of the *MmCu/Zn-SOD* gene in the potato plant genome was analysed by PCR using primer pairs the 35SF and the SODR2. The 35SF primer was located in the 35S CaMV promoter, and the SODR2 primer was located in the middle of *MmCu/Zn-SOD* gene. DNA amplification by PCR to genomic DNA from eight clones of putative transgenic plants produced DNA fragments of about 633 bp, while the genomic DNA from non-transgenic plants did not produce an amplicon. PCR of the pGWB5-MmCu/Zn-SOD plasmid containing the *MmCu/Zn-SOD* gene under the control of the 35S CaMV promoter also produced the DNA fragment of about 633 bp (Figure 3b). This result showed that eight clones of putative transgenic potato cv IPB CP1 plants contained the *MmCu/Zn-SOD* gene under the control of the 35S CaMV promoter integrated into their genome. Because the eight clones were confirmed to contain the *MmCu/Zn-SOD* gene, they were transgenic potato cv. IPB CP1 clones.



(a)



(b)

FIGURE 3 Results of PCR by using DNA of potato cv. IPB CP1 as template. (a) PCR of actin gene, (b) PCR of the *MmCuZn-SOD* gene. 1 kb: 1 kb DNA ladder marker, P: Plasmid pGWB5-MmCuZn-SOD, NT: non-transgenic CP1, CP1S1-8: transgenic CP1 clones.

3.3. Analysis of drought, aluminium, and salinity stress tolerance of transgenic potato plant in vitro

Analysis of tolerance of potato plant to PEG 8000, NaCl, and Al stress in vitro based on the total root length and root number was carried out on five out of eight clones of transgenic plants and one non-transgenic plant as a standard. The growth of stem and roots of both transgenic and non-transgenic plants was inhibited by the stress of 10% PEG 8000, 2 mM Al, and 125 mM NaCl, separately (Figure 4). The inhibition of growth was started seven days after cultivation. Abiotic stress inhibits plant growth and development and reduces plant productivity by damaging cell physiology and biochemical processes through increased ROS synthesis (Gill et al. 2015).

Analysis of potato plant tolerance to drought stress with 10% PEG 8000 showed that transgenic potato plants had more roots than non-transgenic plants. The total root length of transgenic plants was also higher than that of non-transgenic ones. This result indicates that transgenic plants were more tolerant to drought stress than non-transgenic ones. CP1S5 and CP1S6 clones were the highest tolerant to drought.

Analysis of potato plant tolerance to abiotic stress of 10% PEG 8000, stress of 2 mM $AlCl_3.6H_2O$ pH 4.0, and stress of 125 mM NaCl, separately, showed that the transgenic potato clones had higher number of roots than the non-transgenic clones. Under these stresses, transgenic clones also had higher total root lengths than non-transgenic clones (Table 2). This result indicated that the transgenic clones containing the *MmCu/Zn-SOD* gene under the control of the 35S CaMV promoter were more tolerant to drought stress, aluminium and low pH stress, and salinity stress than the non-transgenic clone, although there were variations in tolerance levels among transgenic plants. Under drought stress, clone CP1S5 and clone CP1S6 were most tolerant, while under aluminium and low pH stresses, clone CP1S6 was the most tolerant, and under salinity stress, CP1S2 and CP1S5 transgenic clones were the most tolerant (Table 2). The difference in tolerance levels among the transgenic clones was probably caused by differences in the expression of the *MmCuZn-SOD* gene. Differences in transgene expression in transgenic plants were caused by random site integration of T-DNA containing transgenes, differences in the copy number of transgenes inserted in the plant genome, occurrence of RNA silencing, and regulation by regulatory sequences (Butaye et al. 2005). The expression level of the transgene inserted in euchromatin is different from that of the transgene inserted in heterochromatin. In general, clone CP1S5 and CP1S6 were more tolerant to abiotic stresses than other transgenic clones, probably caused by the expression level of *MmCu/ZnSOD* gene in clone CP1S5 and

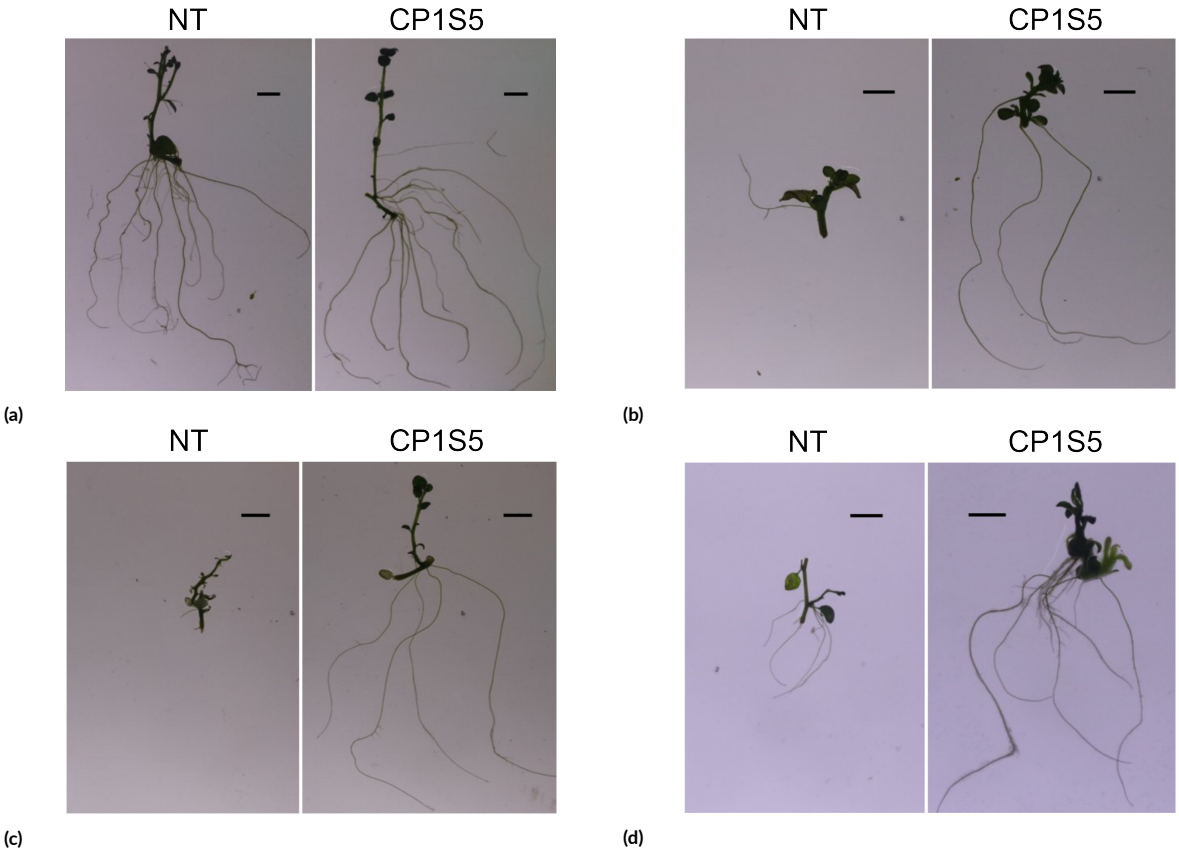


FIGURE 4 In vitro assay of transgenic potato cv. IPB CP1 under various stresses. (a) Without stress, (b) Drought stress by 10% PEG 8000, (c) Aluminium stress by 2 mM Al pH4, d) Salinity stress by 125 mM NaCl. NT: IPB CP1 non-transgenic, CP1S5: transgenic CP1S5 clone. Bar= 1 cm.

TABLE 2 Comparison of total root length and root number of transgenic and non-transgenic potato cv. IPB CP1 plants under various stresses

Clones	125 mM NaCl		2 mM Al, pH4		10% PEG 8000	
	Total Root Lenght (cm)	Root Number	Total Root Lenght (cm)	Root Number	Total Root Lenght (cm)	Root Number
CP1	2.79d	1.08c	1.18e	1.33d	7.49c	2.83c
CP1S2	20.28a	2.58a	18.14d	4.83ab	11.51b	4.58b
CP1S3	13.76b	1.83b	28.76c	4.33bc	11.93b	5.00b
CP1S4	14.78b	2.33ab	30.09bc	4.17c	14.65ab	5.83a
CP1S5	19.23a	2.83a	32.02b	3.67c	15.63a	5.83a
CP1S6	9.55c	1.58b	36.56a	5.17a	16.66a	6.33a

clone CP1S6 being higher than in other transgenic clones. Therefore, further investigation of the expression level of *MmCu/ZnSOD* gene is essential to be carried out.

Drought, aluminium and low pH, and salinity stresses are the main factors that affect the decrease in crop production. These stresses impact the inhibition of root elongation (Yang et al. 2012; Yan et al. 2016). Abiotic stress in plants can lead to the formation of ROS. SOD plays an essential role in protecting plants from the toxic effects of ROS produced during regular cellular metabolic activity or under environmental stress (Wang et al. 2016). SOD enzymes have been widely used to increase plant tolerance to abiotic stresses. SOD expression increased when exposed to drought stress, salinity, and aluminium so that plants were able to maintain root growth and root number when under stress (Wang et al. 2005; Cartes et al. 2012; Houmani et al. 2016). Overexpression of SOD also increased the net photosynthetic rate in potato plants under drought stress (Pal et al. 2013). Transgenic sugarcane (Saccharum officinarum) containing *MmCu/Zn-SOD* gene under the control of 35S CaMV promoter is more tolerant than non-transgenic one to acidic soil containing a high concentration of aluminium (Damayanti et al. 2017). Therefore, this study revealed that SOD increased the tolerance of potato plants to drought, aluminium and salinity stresses.

4. Conclusions

The transgenic potato cv. IPB CP1 containing the *MmCu/Zn-SOD* gene under the control of 35S CaMV promoter has been successfully established. PCR analysis confirmed that eight hygromycin-resistant transgenic potato plants contained the *MmCuZn-SOD* gene and the 35S CaMV promoter. In vitro abiotic stress tolerance assay conducted on five transgenic clones showed that all transgenic clones were more tolerant to drought, aluminum, and salinity stresses than a non-transgenic clone.

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Authors' contributions

M, S, M, AT designed the research. M carried out the laboratory work. M, S analyzed the data. M and S wrote the manuscript. All authors read, revised and approved the final version of the manuscript.

Competing interests

The authors declare no competing interest.

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