Identification of Drought Tolerant Related Insertional Mutant Lines Using PEG 6000

Satya Nugroho, Vincentia Esti Windiastri, Dwi Widyajayanti and Carla Frieda Pantouw
Research Center for Biotechnology-Indonesian Institute of Sciences (LIPI)
Jl. Raya Bogor Km 46, Cibinong 16911, Indonesia
e-mail: nugroho_satya@yahoo.com

ABSTRACT

Drought is one of the most important abiotic stresses in rice (*Oryza sativa*) productivity. The development of drought tolerant cultivars are therefore highly desireable. We have developed insertional mutant based on the Japonica rice cv Nipponbare rice by transposons Ac/Ds insertions containing activation-tag and gene trap. Screening of the mutant population for drought tolerant related phenotypes is of our priority. The screening protocol based on PEG 6000 has been developed and was being used to screen 70 mutant lines to characterize their responds to the treatment based on different parameters (number of leaf, total weight, plant height, root length and number of germinating seeds). These characters were used to score the Degradation Index and Vigour Index. Results showed varrying responds of the lines to the osmotic presure. Some lines showing a good performance indicated by lower Degradation Index and higher Vigour Index have been identified. Some inconsistencies in the performances scored by both indices were thought to be due to seed quality.

Keywords: *Oryza sativa*, insertion mutant, drought, PEG 6000, Degradation Index, Vigor Index.

INTRODUCTION

Drought stress is one of the major limitations to rice production. Improving drought tolerance is one of the most difficult tasks for cereal breeders, due to the complex strategies adopted by plants to combat drought stress depending on the timing, severity and stage of crop growth. Drought stress induces a range of physiological and biochemical responses in plants, which include stomatal closure, repression of cell growth and photosynthesis, and activation of respiration (Shinozaki and Shinozaki, 2007). Plants respond and adapt to water deficit at both the cellular and molecular levels. This can be achieved for instance by the accumulation of osmolytes and proteins specifically involved in stress tolerance. Drought stress most severely affect rice plant during generative stage and considered as one of the main causes of crop loss due to abiotic stress. Many researches on rice drought tolerant improvement have been described, however until recently not many research has been applied to the field yet.

Rice insertional mutant populations have been generated in Japonica rice cv. Nipponbarre using *Agrobacterium* transformation involving T-DNA carrying the two-component *Ac/Ds* transposon system, to enable fast development of large mutant population (Nugroho et al, 2006; 2007a; Jeon et al, 2000; Jung et al, 2003; Hiei et al, 1994). Two construct sysstem were used; harboring gene trap and activation tag, respectively. The activity of the *Ds* transposon to transpose in the presence of *Ac* transposon have been analized and confirmed (Nugroho et al, 2007b). Stable *Ds* mutant have been screened and determined using the available molecular markers (*GFP*, *hpt*, *bar*

and *gusA*). These stable mutants can be used for phenotypic screening.

The advantages of the gene knockout/activation tagging approach for functional genomics include: 1) disruption of gene function leading to loss-of-function mutations, 2) activation of gene expression causing gain-of function mutations, 3) the technique being a direct way to determine the function of a gene product in situ, and 4) the inserted marker being available for subsequent identification of disrupted genes. The mutant population are phenotipically screened for traits related to drought respons and tolerant; such as tillering, weight, leaf quantity, leaf shape and root length.

Polyethylen glycol (PEG) is a chemical commonly used to adjust osmotic pressure. It has been used to induce drought stress like environment, such as in wheat (Blum et al, 1980; Almansouri et *al*., 2001), pea (Sanchez et *al.*, 2004), cotton (Sulistyowati, 2009), maize (Bruce et *al*., 2002), soy bean (Bouslama dan Schapaugh, 1984), rice. PEG 6000 (Mr $\geq$ 6000) is small enough to effect osmotic potensial but too large to absorp by plant. This property makes PEG 6000 the most potensial agent to mimic drought stress in labs (Michel and Kaufmann, 1973; van de Berg dan Zeng, 2006). Initial screening of insertional mutant population generated by using the gene-trap and activation tag constructs for drought related phenotypes using PEG 6000 is described.

MATERIALS AND METHODS

Plant materials

Rice cv Nipponbare insertional lines (70 lines) generated previously were used (Nugroho et al, 2006; 2007a), consisting of 50 activation tag lines and 20 gene trap lines.

Drought related phenotype screening

Seeds were washed with sterilized water 3 times followed by application of 70% ethanol for 5 minutes. The seeds were then sterilized in 20% sodium hypochloride solution containing 3 drops of Tween 20 for 30 minutes. The seeds were then washed 3 times in sterilized water. Sterilized seeds were germinated in open bottles (10 seed/bottle) containing 25 ml Yoshida solution (Yoshida et al, 1976) containing 200 g/L PEG 6000 in the jam bottles and placed inside closed transparent plastic boxes. Aeration was provided by holes on each sides of the box. To maintain relative humidity, the bottles were kept inside aerated closed transparent plastic boxes containing NaCl saturated water. The solution media was replaced every week.

Droughtrelated phenotypes scoring

Drought related phenotypes were scored 21 days after the drought treatment, including number of leaf, total weight, plant height, root length and number of germinating seeds. The reduction in plant performance compared to control plants was measured using the degradation and vigor indices using the following formula:

$$\text{Degradation Index} = 1 - \frac{\text{Treatment score}}{\text{Control score}}$$

Vigor Index = Jumlah perkecambahan x (plant height + root length)

The degradation indices for activation tag and gene traplines were presented separately, for the ease of future analysis.

RESULTS AND DISCUSION

Previousely, the PEG 6000 concentration for screening had been optimized (unpublished data). Treatment with 200 g/L PEG 6000 on rice cv Nipponbare showed reduce growth but maintain good germination rates as compared to 250 g/L PEG 6000 which severely reduced germination, or 100 g/L which showed germination similar to untreated control.

Responds of gene-trap and activation tag insertional mutants to 200 g/L PEG 6000

Scoring using number of leaves, plant height, root length, and total weight, variations among mutant lines germinated in 200 g/L PEG 6000 were observed. Some lines showed reduced growth under the osmotic stress, however some others showed growth similar to untreated control. Those phenotypic variations might indicate different mutations. The degradation index data using each parameter for the activation tag lines were shown in Table 1.

Tabel 1. Degradation index of the activation tag lines on different parameters. DI, Degradation Index; NB, Nipponbare; Salumpikit, drought tolerant lines; IR20, drought sensitive lines.

Degradation Index							
Number of Leaves		Total Weight		Plant Height		Root Length	
Genotype	DI	Genotype	DI	Genotype	DI	Genotype	DI
T3 PMO VI 30 1A-24-8-11	32,26	T3 PMO III 4-4C-22-1-12	24,39	T3 PMO III 4-4C-22-1-12	36,21	T3 PMO III 4-4C-22-1-12	-2,81
T3 PMO III 4-4C-22-1-12	40,89	T3 PMO VI 30 1A-24-8-11	60,69	T2 PMO VI 30 1A-21	76,67	T2 PMO VI 30 1A-21	17,98
T3 PMO 30 1A 42-3	67,85	T2 PMO VI 30 1A-21	62,29	T3 PMO VI 30 1A-24-8-11	76,87	T3 PMO VI 30 1A-24-8-11	42,51
T2 PMO VI 30 1A-21	50,46	T3 PMO III 4-4C-22-1-11	67,68	T3 PMO VI 30 1A-15-1	83,27	T3 PMO III 4-4C-22-1-11	71,30
T3 PMO III 4-4C-22-1-11	58,33	T3 PMO VI 30 1A-15-1	77,15	T3 PMO III 4-4C-22-1-11	83,65	T3 PMO VI 108B-28-3	100,00
NB	46,67	NB	69,18	NB	88,59	NB	25,69
Salumpikit	55,56	Salumpikit	73,64	Salumpikit	92,89	Salumpikit	84,69
IR20	69,70	IR 20	79,84	IR 20	96,86	IR 20	88,76

Tabel 1 shows only the top 5 degradation index for the 4 parameters (number of leaves, total weight, plant height and root length). Looking at the degradation index, lines on the top 5 had degradation index lower than the tolerant control (Salumpikit), indicated that they were more tolerant to drought. The lower the degradation index, the better the plant growth. This can also be interpreted as the more tolerant the lines to PEG 6000, or in other word the more tolerant the lines to drought stress. The higher the degradation index, the less tolerant the lines to drought stress.

The results showed that line T3 PMO III 4-4C-22-1-12 was lowest in all parameters except on number of leaves. The lines grew relatively well under PEG 6000 stress. The root length of line T3 PMO III 4-4C-22-1-12 developed exceptionally well (Index degradation -2.81) compared to the tolerant control lines (Salumpikit, 84.69). Root length is a vital trait against drought stress in plant cultivars. The plant embryo grows at germination and develops radicles that become the primary roots which penetrate into the soil. Hypocotyl is then emerges and lifts the growing tip above the ground. The hypocotyls are the primary organ of extension which will develop into the stem. Under drought stress condition, the root develops faster than the hypocotyls to acclimatize to the drought stress. Therefore, the growth of radicle and hypocotyls should reflect the adaptability of plant to drought stress (Zhu et al; 2006). Thus, root plays a role in plant survival during drought stress and drought tolerant can be characterized by extensive root growth and reduction of shoot growth.

Line T3 PMO VI 30 1A-24-8-11 also showed similar consistencies as line T3 PMO III 4-4C-22-1-12 in drought responses. Eventhough all lines showed less degradation and can be considered as more tolerant to drought than the control,

lines T3 PMO VI 30 1A-24-8-11 and T3 PMO III 4-4C-22-1-12 are strongest candidate for drought tolerant lines.

Table 2. Degradation index of the gene trap lines on different parameters. DI, Degradation Index; NB, Nipponbare; Salumpikit, drought tolerant lines; IR20, drought sensitive lines.

Degradation Index							
Numbr of Leaves		Total Weight		Plant Height		Root Length	
Genotype	DI	Genotype	DI	Genotype	DI	Genotype	DI
F3 PUR X 21-2A-7-15	20,05	F3 PUR X 21-2A-7-17	24,21	F4 PUR VIII 4-1F2-1-112-1	43,71	F3 PUR X 21-2A-7-15	-36,31
F3 PUR X 7-3A-3-16	22,16	F3 PUR X 21-2A-7-10	41,97	F3 PUR X 21-2A-7-15	51,29	F3 PUR X 7-3A-3-3	-24,02
F3 PUR IX 47-1B-8-3	24,76	F3 PUR X 21-2A-7-15	56,68	F3 PUR X 21-2A-7-16	57,35	F3 PUR X 21-2A-7-10	-14,07
F3 PUR X 21-2A-7-10	25,71	F3 PUR X 81-2B-2-1	58,47	F3 PUR X 81-2B-2-1	63,49	F4 PUR VIII 4-1F2-1-112-1	-12,69
F3 PUR X 21-2A-7-17	26,57	F4 PUR VIII 4-1F2-1-112-1	58,63	F3 PUR IX 47-1B-8-3	69,60	F3 PUR IX 47-1B-8-3	-1,76
F3 PUR X 21-2A-7-16	27,84	F3 PUR X 21-2A-7-16	59,79	F3 PUR IX 49-2C-1-19-16	69,95	F3 PUR X 21-2A-7-16	13,90
F3 PUR X 81-2B-2-1	28,57	F3 PUR IX 47-1B-8-3	60,82	F3 PUR X 21-2A-7-17	70,18	F3 PUR X 21-2A-7-17	15,18
F4 PUR VIII 4-1F(2)-1-121-7	30,41	F3 PUR X 7-3A-3-3	61,49	F3 PUR X 7-3A-3-16	72,24	F4 PUR VIII 4-1F(2)-1-121-7	21,85
F3 PUR X 21-2A-7-14	32,43	F3 PUR X 21-2A-7-14	63,35	F4 PUR VIII 4-1F(2)-1-121-7	72,40	F3 PUR X 21-2A-7-14	27,59
F4 PUR VIII 4-1F2-1-112-1	33,92	F3 PUR X 7-3A-3-16	63,73	F3 PUR X 7-3A-3-3	72,68	F3 PUR VIII 5-1F-1-4-8	28,07
NB	46,67	NB	69,18	NB	88,59	NB	25,69
Salumpikit	55,56	Salumpikit	73,64	Salumpikit	92,89	Salumpikit	84,66
IR 20	69,70	IR 20	79,84	IR 20	96,86	IR 20	88,76

Unlike the activation tag lines, the results from the gene trap lines were more diverse. The lowest degradation indices were not shared by the same lines except for line F3 PUR X 21-2A-7-15. Eventhough line F3 PUR X 21-2A-7-15 performed well in number of leaves and notably in root length, it did not perform as well in the other parameters. However, in total, line F3 PUR X 21-2A-7-15 together with lines F3 PUR IX 47-1B-8-3, F3 PUR X 21-2A-7-17, F3 PUR X 21-2A-7-16, F4 and PUR VIII 4-1F2-1-112-1 showed lower degradation indices in all categories than the control line. Therefore those lines can be considered as drought tolerant candidates. Other lines (F3 PUR X 21-2A-7-10, F3 PUR X 7-3A-3-16 and F3 PUR X 7-3A-3-3) showed better performance in 3 categories than the control line.

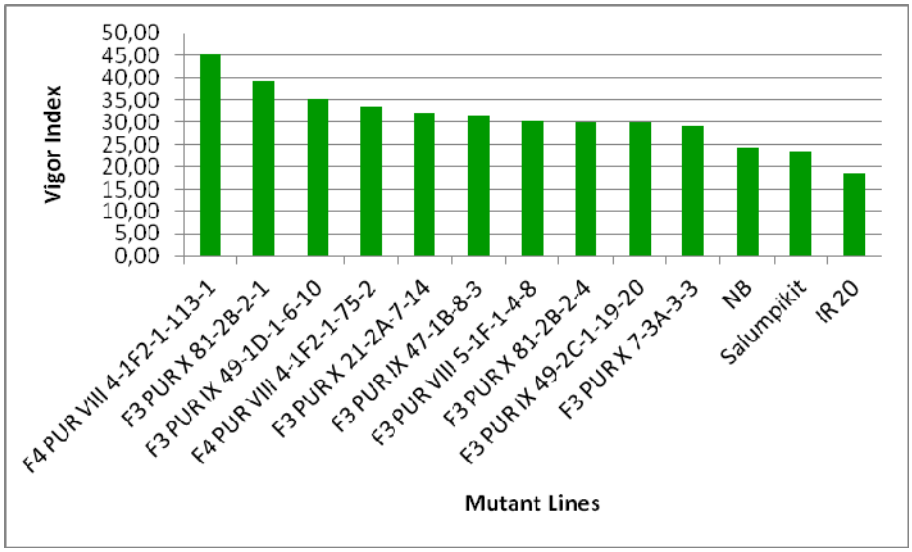
Eventhough the 3 lines might be considered as drought tolerant candidates in the 3 categories compared to the control lines, candidates with stronger character in the root length will be preferable as drought tolerant candidates (F3 PUR X 7-3A-3-3 and F3 PUR X 21-2A-7-10).

Vigor Index

Water availability is one of the most important environmental factors influencing seed germination as it triggers the germination process. Inadequate moisture availability is one of the factors that affect germination failure due drought stress (Ramesh and Devasenapathy; 2007). Water stress limits seedling growth by delaying its beginning or decreasing the final germinability. Seed germination and early seedling growth are considered as most critical phases for seed establishment, determining successful crop production (Uniyal et al; 1998). Seedling vigor test under drought imposed environment using PEG 6000, will tell us if seed of particular lines are more tolerant or sensitive to drought stress.

Ten gene trap lines with the highest seedling vigor index are presented in Figure 1. The decrease in vigor index was clearly shown between the untreated and PEG 6000 treated, which was due to the osmotic stress. The stress induces slower germination. Among the gene trap lines tested, line F3 pUR VIII 5-1F-1-4-8 appeared to have high index vigor both in the treated and untreated media. The result might indicate that line F3 pUR VIII 5-1F-1-4-8 poses a drought tolerant traits. However, interestingly, this line did not appear in the scoring for the degradation index (Table 2). This may be due to the complex nature of drought stress responses. Line F3 PUR X 21-2A-7-16 showed lower degradation index (Table 2) and it also appear to have higher vigor index under PEG 6000 treatment (Figure 1 B). Data also showed that this lines has vigor index similar to Nipponbare lines under no PEG 6000 treatment (data not shown). Therefore, F3 PUR X 21-2A-7-16 might be a good candidate for a drought tolerant line. Although in terms of degradation index it did not perform as well as other lines, compared to control lines drought tolerant line (Salumpikit) it performed better.

A.



B.

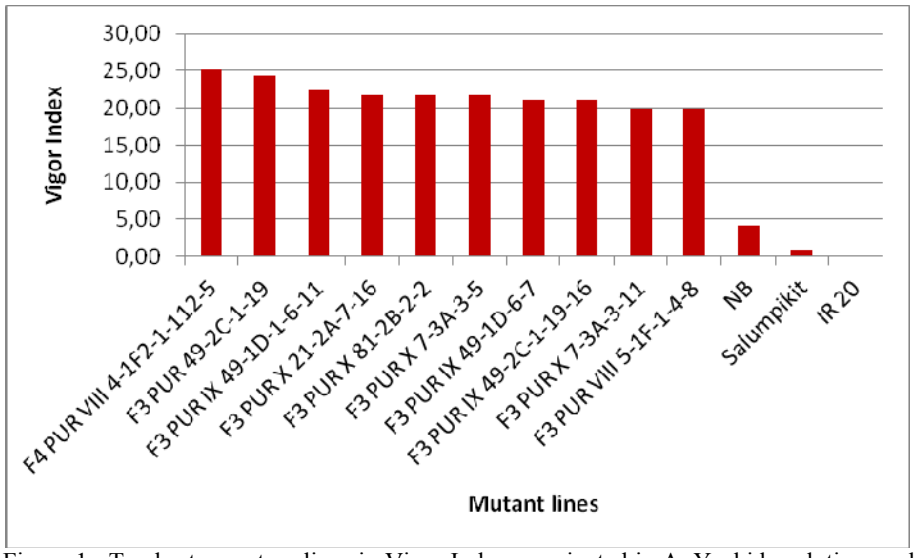
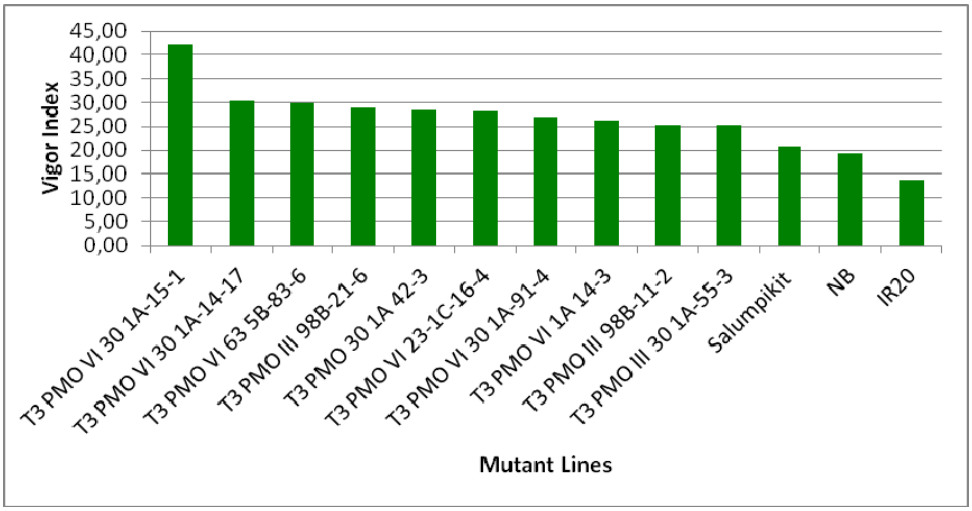


Figure 1. Ten best gene trap lines in Vigor Index germinated in A. Yoshida solution, and B. Yoshida Solution with 200 g/L PEG 6000

Vigor Index analysis of the activation tag lines showed that two lines T3 pMO VI 30 1A-14-7 dan T3 pMO VI 23-1C 16-4 were consistently appear to have good score both in the PEG 6000 treated and untreated media (Figure 2). However none of them appear in the Degradation Index results (Tabel 1). Line T3 PMO III 4-4C-22-1-12, which performed consistently well in all criteria in the degradation index did not score well in the vigor index. It scored below the Nipponbare control lines. Line T3 PMO VI 30 1A-24-8-11, which also scored well in the degradation index (although not as well as T3 PMO III 4-4C-22-1-12), gave sligthly better score for vigor index (data not shown). However it is not as well as the Nipponbare control line. The inconsistency of the results between Degradation Index and Vigor Index may due to several reasons. One of them being the seed quality. Seed storage and treatment play roles in seed quality. In this case seeds were collected from different batches harvested from different times and seasons, which may influence seeds performance.

A.



B.

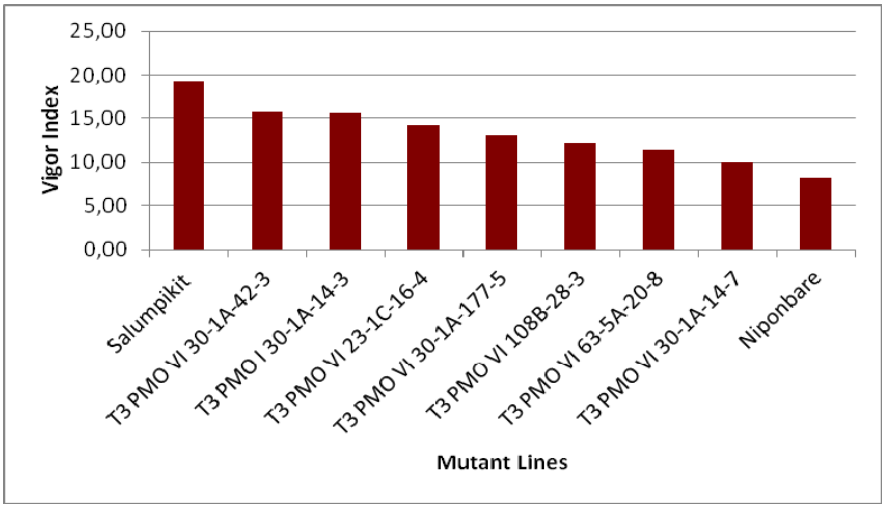


Figure 2. Ten best activation tag lines in Vigor Index germinated in A. Yoshida solution, and B. Yoshida Solution with 200 g/L PEG 6000.

REFERENCE

Almansouri M, Kinnet JM and Lutts S. 2001. Effect of salt and osmotic stress in durum wheat (*Triticum durum* Desf.) Plant & Soil 231: 243 – 254

Blum A, Sinmena B and Ziv O. 1980. An evaluation of seed and seedling drought tolerance screening tests in wheat. Euphytica 29: 727-736

Bouslama, M and WT Schapaugh, Jr. 1984. Stress Tolerance in Soybeans. I. Evaluation of Three Screening Techniques for Heat and Drought Tolerance. Crop. Sci. 24:933-93

Bruce BW, Edmeades GO, and Barker TC. 2002. Molecular and physiological aproaches to maize improvement in drought tolerance. J. Ext. Botany 53(366): 13-25

Hiei Y, Ohta S, Komari T, Kumashiro T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T DNA. *Plant J.* 6: 271-282.

Jeon JS, Lee S, Jung KH, Jun SH, Jeong DH, Lee J, Kim C, Jang S, Yang K,

Nam J, An K, Han MJ, Sung RJ, Choi HS, Yu JH, Choi JH, Cho SY, Cha SS, Kim SI, An G. 2000. T-DNA insertional mutagenesis for functional genomics in rice. Plant J. 22: 561-570.

Jung KH, Hur J, Ryu CH, Choi Y, Chung YY, Miyao A, Hirochika H, An G. 2003. Characterization of a rice chlorophyll-deficient mutant using the T-DNA gene-trap system. Plant Cell Physiol. 44: 463-472.

Michel BE, Kaufmann MR. 1973. The osmotic potential of polyethylene glycol 6000. Plant Physiol. 51:914–916

Nugroho S, Trijatmiko KR, Rachmawati S, Zannati A, Purwantomo S. 2006. Upaya pengembangan populasi mutagenic lines padi pembawa *activation tag* dengan transposon *Ac/Ds* melalui transformasi dengan *Agrobacterium*. Prosiding Seminar Nasional Bioteknologi. 15-16 November 2006 . p 236-242.

Nugroho S, Mulyaningsih ES, Astuti D, Pantouw C. 2007a. Upaya pengembangan populasi padi mutan dengan mutasi insersi transposon *Ac/Ds* pembawa *gene-trap* untuk pencarian gen-gen penting dari padi. Prosiding Seminar pengembangan dan optimalisasi produksi komoditas tanaman pangan, hortikultura perkebunan dan bioenergy. P. 105-110.

Nugroho S, Trijatmiko KR, Zannati A, Indrayani S. 2007b. Analisa aktivitas transposon *Ds* pembawa *activation-tag* pada generasi T2 dalam upaya pengembangan populasi padi mutan untuk pencarian gen-gen bermanfaat. Prosiding Seminar pengembangan dan optimalisasi produksi komoditas tanaman pangan, hortikultura perkebunan dan bioenergy. P. 111-114.

Ramesh T and Devasenapathy P. 2007. Naturalresources management on sustainable productivity of rainfed Pigeonpea (Cajanus cajan L.), Res. J. Agric. Biol. Sci. 3(3): 124-128.

Sanchez FJ, EF de Andres, J.L. Tenorio and L. Ayerbe. 2004. Growth of epicotyls, turgor mantenance and osmotic adjusment in pea plants (*Pisum sativum* L.) subjected towater stress. Field Crops Research 86: 81 – 90.

Shinozaki K, Yamaguchi-Shinozaki K. 2007. Gene networks involved in drought stress response and tolerance. J Exp Bot. 58(2):221-7.

Sulistyowati, E. 2009. Pemanfaatan teknologi transgenik untuk perakitan varietas unggul kapas tahan kekeringan. Perspektif 10 (2): 96 – 107.

Uniyal RC and Nautiyal AR. 1998. Seed germination and seedling extension growth in Ougeinia dalbergioides benth. Under water and salinity stress, New Forests 16: 265-272.

Van de Berg L and Zeng YJ. 2006. Response of South African indigenouse grass to drought stress induced by polyethylene glicol (PEG) 6000. South African Journal of Botany 72: 284 – 286

Yoshida, S. 1981. Fundamental of rice crop science. IIRRI. Los Banos, Philippines. 269 p.

Zhu J, Kang H, Tan H and Xu M. 2006. Effects of drought stresses induced by polyethylene glycol on germination of Pinus sylvestris var. mongolica seeds from natural and plantation forests on sandy land. J. For. Res. 11: 319-328.