Electronic Journal of Plant Breeding, 8(4): 1293-1297 (Dec 2017)
ISSN 0975-928X
DOI: 10.5958/0975-928X.2017.00185.5

Research Note

Expression analysis in response to heat stress in moth bean (*Vigna aconitifolia* (Jacq.) Marechal)

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(Received: 16 Nov 2017; Revised: 10 Dec 2017; Accepted: 28 Dec 2017)

Abstract
Among abiotic stresses, heat stress has a large negative impact upon agricultural productivity worldwide. Moth bean (*Vigna aconitifolia* (Jacq.) Marechal) is attracting a great deal of attention due to its drought and heat stress tolerance nature although genotypic variability exists. Genotypes of moth bean were subjected to heat stress to reveal the pattern of expression of genes. Many heat shock proteins (HSPs) and Super oxide dismutase (MnSOD) are differentially regulated in plants subjected to heat stress. Twenty days-old moth bean seedlings were exposed to a temperature of 55°C for 30 minutes. Total RNA was extracted from stressed and control plants, proceed for qRT-PCR analysis. Heat shock protein (HSP70) and MnSOD were significantly up-regulated in Jwala, Jadia, Marumoth and RMO 40 genotypes of moth bean and down-regulated in genotypes with accession numbers, IC 121051, IC 36392, IC 140725 and IC 472257. This study would help in identifying heat tolerant varieties in moth bean and related *Vigna* species for future breeding programmes.

Key words
Moth bean, heat stress, expression analysis, heat shock proteins

Abiotic stresses are those non-living environmental factors that can harmfully affect the growth, productivity and yield of the crops. Drought, salinity and extreme temperatures are the major abiotic stress factors that negatively affects the crops worldwide besides nutritional deficiencies and disorders like deficits of inorganic ions (nitrogen, cadmium, potassium, phosphorus) also harmful factors for plants growth and productivity. All kinds of abiotic stresses are inter-related and they cause morpho-physiological, biochemical and molecular changes in plants that negatively affect the plant productivity and yield (Naya *et al*. 2007). Heat stress is one of the most important abiotic stresses that causes oxidative stress and generates reactive oxygen species (ROS). Temperature plays a crucial role in plant growth, development and yield (Mitter *et al*. 2012). Plants respond to heat stress by altering the expression of several genes and andn thereby altering various cellular, physiological and biochemical processes towards adaptation.

Moth bean (Vigna aconitifolia) known to be a stress hardy legume crop, is an important source of grain and fodder. It is commonly known as moth bean, matki bean, matki and dew bean belonging to Fabaceae family having a chromosome number $2n = 22$. In India moth bean is grown on total of 9.28 lakh hectares and 2.87 lakh tonnes of moth bean production was recorded during year 2012-2015 (Ministry of agricultural). Rajasthan is the main moth bean growing state, contributing about 96% in area and 94% in production (Department of Agriculture, Rajasthan 2015) Moth bean a short-day crop is one of the most drought and heat tolerant cultigens among Asian *Vigna* (Tomooka et al. 2006) It increases land productivity and can be used as a second crop as well which follows the main crop. The most favourable temperature for production of moth bean is between 24-42°C but it can survive at temperatures up to 40-45°C and indicated an inducible nature of thermo-tolerance in moth bean with genetic variations for level of induction (Sharma *et al.,* 2014). Moth bean is an important source of proteins, minerals and vitamins. In the present study, the pattern of gene expression analysis in moth bean genotypes was assessed under control and heat stress condition.

In the present study, 200 accessions were grown in the field under normal growth condition. All the accessions were collected from different parts of Maharashtra and Rajasthan. Morphological observations were recorded in each moth bean genotype for plant height, number of branches per plant, number of clusters per plant, cluster length, number of pods per plant, pods per branch, pod length and 100- seed weight. Also, yield attributing parameters like numbers of pods per plant, number of pods per cluster, number of cluster per plant, number of seeds per pod and 100 -seed weight were observed.A total of 10 moth bean accessions were selected from 240 accessions on the basis of morphological parameters *viz.* Jwala, Jadia, Marumoth, RMO 40, IC 121051, IC 36392, IC 39702, IC 472257, IC 140725, IC 36157. Seeds were sown at NBPGR in three replicates in vermiculite/ sand mixture into small pots inside a growth chamber at 25°C under 16 h light and 8 h darkness during 2016. Twenty-day-old plants with similar size were selected and subjected to heat stress at 55°C for 30 minutes in the oven.Total RNA was isolated from control and stressed leaves in two technical replicates using the RNAeasy mini kit (Qiagen, USA). First strand cDNA was synthesized with 1 µg of the total RNA using cDNA reverse transcription kit (Invitrogen, USA). Batch Primer3 program was used to design primers for stress responsive genes from transcriptome data of moth bean (Unpublished) (Table 1). Real time PCR was performed in Light Cycler 480 (Roche, Switzerland) using SYBR Green qPCR Master Mix (KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal). The qPCR program consisted of an initial denaturation step at 95°C for 3 min, followed by 40 cycles of 95°C for 30s, 48°C for 60s and 72°C for 20s. For each primer, melting curves were analyzed for a single peak to confirm the specificity of the primers. Relative expressions of all genes were calculated by $_{\Delta\Delta}$CT method (Livak *et al.* 2001).

Morphological parameters such as seed yield per plant, number of clusters per plant, number of pods per plant, number of clusters per plant, number of pods per cluster and 100- seed weight (Table 2) representing easiest selection for crop improvement. It indicates that the presence of genetic variability and selection of material is suitable. For the selection of tolerant and susceptible varieties, conventional and trait based approaches have been used and selected ten genotypes of moth bean and further proceeded for gene expression analysis. Plant height observed ranged from 16.74 cm (IC-121051), 20.82cm (IC-39702) to highest ranged 37.98 cm (Jwala), 27.3 cm (RMO-40) and 23.54 cm (Jadia) in moth bean genotypes. The number of branches per plant observed highest ranged were 8.6 (IC-36392), 6.8 (Jadia) and 5.6 (Jwala) while lowest ranges noted were 4.4 (IC-36157) and 5 (IC-121051) in moth bean genotypes. The number of clusters per plant varied from highest 14.8 (Jadia), 14 (Jwala), 13.4 (RMO-40), lowest clusters per plant 6.2 (IC-39702) and 7.2 (IC-121051) in moth bean genotypes. The maximum number of pods per plant were recorded in the highest 41.6 (Jadia) and 41 (Jwala) while lowest pods per plant observed in 15 (IC-121051), 15.8 (IC-36157) and 17.0 (IC-39702) in moth bean genotypes. The seed yield per plant were recorded in the highest per plant 30.87 (Jwala), 28.7 (Jadia), 17.85 (IC-36157) while lowest ranged 4.39 (IC-36392), 10 (IC-39702), 11.3 (IC-472257) were observed in moth bean genotypes. The maximum seed weight was recorded in the highest per plant 4.01gm (RMO 40), 3.61gm (Jwala), 3.51gm (Jadia), 3.3gm (IC-36157) and lower ranged were noted in 1.3gm (IC-140725), 1.39gm (IC-39702), 1.58gm (IC-472257) in moth bean genotypes. Two differentially expressed genes (HSP70, MnSOD) with known functions were selected for real time analysis. The relative expression study of differentially expressed genes can help in understanding the key genes and mechanism involved in heat stress tolerance in moth bean. Hence, each experiment needs the identification of the most stable reference gene for valid data normalization (Vandesompele *et al*. 2002). The RT-PCR analysis indicated up and down-regulation of genes after heat stress treatments in different genotypes of moth bean. Relative gene expression of HSP70 and MnSOD of heat stressed plants versus the control plants was calculated by $_{\Delta\Delta}$CT method. The family of heat shock proteins with the molecular weight of 70kDa is widely known as HSP70 and acts as molecular chaperones and found in plant as well as animals. In the present study tolerant genotypes Jwala, Jadia, Maru-moth and RMO 40 showed significant up-regulation (2.5, 2.0, 1.9, 3.2 fold respectively) of HSP70 under heat stress (Figure 1). Highly susceptible genotypes viz., IC 39702, IC 140725 and IC 472257 showed significant down-regulation of HSP70 under heat stress. The expression profile of HSP70 revealed that this gene is highly expressed in response to heat stress in moth bean. Transcript of Hsp70 gene are expressed constitutively and during development and the expression is enhanced significantly under various stress conditions in rice (Sarkar *et al*. 2013).

Similar results also found in bentgrass and Dichanthelium lanuginosum grass, expression of HSP70 in both roots and shoot before heat stress and increase the expression of HSP70 after heat stress (Wang *et al*. 2014; Al-Niemi *et al.* 2002). In *Arabidopsis* also found higher expression level of HSP70 upon heat stress (Sung *et al.* 2003). In all varieties of alfalfa plant the HSP70 level in both control and heat stressed condition varied and also observed the early recovery of plant after heat stress (Ferradini *et al*. 2015). Super oxide dismutase (SOD) has a critical role in the survival of plants under stressed environment (Gill and Tuteja 2010). SODs are classified into 3 groups on the basis of metal cofactor. Our findings shows the significant up-regulation of MnSOD in Jwala, Jadia, Maru-moth and RMO 40 and IC 36157 (2, 1.5, 2.2, 4, 1.9 fold respectively) but highly down-regulated in susceptible genotypes IC 39702, IC 472257, IC 140725, IC 36392 (Fig.1). In Rice found the higher expression of a MnSOD in drought tolerance (Wang *et al.* 2005). Similar expression pattern observed in treated rice cultivars were normalized to their corresponding controls, which were defaulted as 1 under different abiotic stress (Fang *et al*., 2014).

It is concluded from above study that the tolerance and susceptibility of genotypes were also associated by expression studies of two genes *viz. Hsp70* and *MnSOD* involving in stress tolerance mechanism and revealed the moth bean genotypes, Jwala, Jadia, Marumoth, RMO 40 and IC 36157 as heat tolerant which can be used in breeding programs to develop more heat tolerant varieties.

Acknowledgement

The authors thank the Indian Council of Agricultural Research and Director, NBPGR, New Delhi, for the facilities provided for the study.

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Table 1. Details of primers used for real time validation in moth bean

Transcript id	Primer's name	Melting temperature	Sequence
trans_s_2626	MnSOD_F	48	GAATCTCCCATCTCTGGTA
	MnSOD_R		AGTTGAAGAGGCTGTAGTTG
trans_s_4103	HSP70_F	48	CTGATGTCCTTCTTATGCTT
	HSP70_R		TGCTGGTCTCAATGTACTTA
Actin	Actin_F	48	GAAGTATCCTATTGAGCATGGC
	Actin_R		ACCCTTCATATATGGGCACC

Table 2. Mean values of 10 different morphological traits for 10 genotypes of moth bean

Genotypes	Plant Height (cm)	Peduncle Length (cm)	No. of Branches per Plant	No. of clusters / plant	No. of Pods on branches	No. of pods /plants	Pod length (cm)	Pod yield /plant (gm)	Seed yield /plant (gm)	100 seed weight (gm)
Jwala	37.98	10.5	5.6	14	151.6	41	4.4	38.12	30.87	3.61
Jadia	36.54	2.78	6.8	14.8	103.4	41.6	4.44	35.77	28.71	3.51
Marumoth	27.9	7.42	5.4	8.4	64.8	25.8	4.84	24.23	22.91	2.92
RMO 40	27.3	3.44	4.4	13.4	60.8	53.2	4.88	28.06	22.92	4.01
IC121051	16.74	5.42	5	7.2	44.4	15.0	4.1	14.7	12.91	2.12
IC36392	24.2	7.28	8.6	10.4	45	21.8	4.02	16.05	4.39	2.16
IC39702	20.82	7.24	5.6	6.2	44.8	17.0	4.1	17.4	10.01	1.39
IC472257	23.85	8.84	6.2	12.8	97.6	30.4	4.3	26.2	11.34	1.58
IC140725	26.9	4.42	5.8	14.0	79.4	29.6	3.9	24.9	12.85	1.3
IC36157	20.58	7.98	4.4	6.6	40.2	15.8	4.5	15.6	17.85	3.4

Figure 1. Relative expression of stress genes (HSP70, MnSOD) in heat tolerant and susceptible genotypes of moth bean under control and heat stress