Int.J.Curr.Microbiol.App.Sci (2018) *7*(9): 1426-1436

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 7 Number 09 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.709.171

Effect of Mannose Specific Lectins *ASAL* and *GNA* on the Feeding Behavior of BPH (*Nilaparvata lugens stal*.)

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Keywords

Transgenic rice, ASAL, GNA, Feeding behavior, Honeydew

Article Info

Accepted:
10 August 2018
Available Online:
10 September 2018

A B S T R A C T

Rice (*Oryza sativa*) productivity is adversely impacted by numerous biotic and abiotic factors. An approximate 52% of the global production of rice is lost annually owing to the damage caused by biotic factors, of which ~21% is attributed to the attack of insect pests. We have developed transgenic pyramided rice lines, endowed with enhanced resistance to major sap sucking insects, through sexual crosses made between two stable transgenic rice lines containing *Allium sativum* (*ASAL*) and *Galanthus nivalis* (*GNA*) lectin genes. Presence and expression of *asal* and *gna* genes in pyramided lines were confirmed by PCR and western blot analyses. Segregation analysis of F_2 disclosed digenic (9:3:3:1) inheritance of the transgenes. Homozygous F_3 progenies plants carrying *asal* and *gna* genes were identified employing genetic and molecular methods besides insect bioassays. Pyramided lines, infested with brown plant hopper (BPH), proved more effective in reducing insect survival, fecundity, feeding ability besides delayed development of insects as compared to the parental transgenics. Under infested conditions, pyramided lines were found superior to the both the parental transgenics in their seed yield potential. This study also reveals the feeding behavior of the BPH insects on both the pyramided as well as parental transgenic lines and the effect of mannose specific lectins *asal* and *gna* under the control of different promoters CaMV35S and Rss1 on the feeding behavior of BPH. BPH insects fed on GNA transgenic plants showed phloem specific feeding up to 72 h and later switched over to xylem feeding after 72 h. In contrast, BPH insects fed on ASAL transgenic rice plants did not show any difference in the feeding behavior even after 96h. The pyramided lines appear promising and might serve as a novel genetic resource in rice breeding aimed at durable and broad based resistance against hoppers.

Introduction

Rice (*Oryza sativa* L.) is one of the world's most important crops, providing a staple food for nearly half of the global population (FAO, 2004). Almost 90% of the rice is grown and consumed in Asia (Khush and Brar, 2002). Approximately 15% of agricultural produce is lost every year to insects, and consequently farmers spend billions of US$ yearly to

provide effective control using chemical insecticides. Plants have been embattled in a war with the chewing, sucking and piercing insects for millions of years (Zhu-Salzman *et al.,* 2005). The homopteran pests, rice brown plant hopper (*Nilaparvata lugens*), rice green leafhopper (*Nephotettix virescens*) and white backed planthopper (*Sogatella furcifera*) cause severe physiological damage to the rice plants, besides acting as vectors for major viral diseases (Mochida *et al.,* 1979; Saxena and Khan, 1989; Dahal *et al.,* 1997; Foissac *et al.,* 2000). Chemical insecticides provide a simple way to control insect infestation, but use of agrochemicals without effective biosafety rules may lead to both environmental and health problems (Bajaj and Mohanty, 2005). In this context, genetic engineering of rice for insect resistance provides a potent, cost-effective and environment friendly option (Bajaj and Mohanty, 2005).

To develop new strategies for insect resistance crops, different insect-resistance genes, conferring resistance to major pests, have been identified from various sources for transferring them into cultivated crops (Estruch *et al.,* 1997; Gatehouse and Gatehouse, 1998). In different crops, insect resistant transgenic plants were obtained through the introduction of *Bacillus thuringiensis* (Bt) crystal protein (*cry*) genes, plant derived protease inhibitors (PIs) and lectins (Hilder *et al.,* 1987; Boulter *et al.,* 1990; Peferoen, 1992; Wunn *et al.,* 1996; Nayak *et al.,* 1997; Cheng *et al.,* 1998; Datta *et al.,* 1998; Maqbool *et al.,* 2001, Nagadhara *et al.,* 2003, 2004 and Yarasi *et al.,* 2008, 2011).

Lectins are proteins or glycoproteins of non-immune origin with one or more binding sites per subunit, which can reversibly bind to specific sugar segments through hydrogen bonds and Van Der Waals interactions (Lis and Sharon, 1998). Mannose-binding plant lectins have been proved to be promising candidates for the control of homopteran insect pests, not only for different insecticidal mechanisms, but also for their complementarities to Bt toxins and protease inhibitors. Earlier investigation indicated that the snowdrop lectin protein (GNA) isolated from the monocotyledonous plant, *Galanthus nivalis* (snowdrop), belonging to amaryllidaceous family, is toxic to sap-sucking insects of rice when fed in artificial diet. Transgenic plants expressing GNA showed significant entomotoxic effects as evidenced by insect bioassays under controlled conditions (Hilder *et al.,* 1995; Down *et al.,* 1996; Gatehouse *et al.,* 1996; Czapla, 1997; Rao *et al.,* 1998; Foissac *et al.,* 2000; Couty *et al.,* 2001; Nagadhara *et al.,* 2003, 2004). Similarly, bioassays based on artificial-diet-feeding system, using mannose-specific lectin from *Allium sativum* agglutinin (ASA and ASAL), showed antimetabolic effects towards BPH and GLH insects (Powell *et al.,* 1995; Majumder *et al.,* 2004). Transgenic rice expressing ASAL exhibited ample resistance against homopteran insects BPH and GLH (Saha *et al.,* 2006) and for BPH, GLH and WBPH (Yarasi *et al.,* 2008, 2011).

Earlier it was reported that, GNA under the control of Rice sucrose synthase promoter and a Maize ubiquitin promoter, confers resistance towards BPH, despite the different levels of GNA as a proportion of total protein, plants derived from pRSsGNA and pUbiGNA gave similar results in the insect bioassays, suggesting that the phloem-specific promoter was also effective in delivering GNA to the insects (Rao *et al.,* 1998). The expression efficiency of ASAL transgenics in rice was monitored, from two phloem specific promoters, RSs1, rolC and a constitutive CaMV35S promoter, rolC demonstrated to be stronger and more effective for engineering

resistance to phloem limited viruses, than phloem-specific RSs1 promoter and CaMV35S (Saha *et al.,* 2006).

The present study deals with the differential feeding behavior of BPH fed on transgenic rice plants expressing GNA under the control of phloem specific rice sucrose synthase promoter (Rss1) and transgenic rice plants expressing ASAL under the control of CaMV35S constitutive promoter. BPH insects fed on GNA trangenic plants showed phloem specific feeding up to 72 h and later switched over to xylem feeding after 72 h. In contrast, BPH insects fed on ASAL transgenic rice plants did not show any difference in the feeding behaviour even after 96h. This report also demonstrates that the mannose specific lectins, GNA and ASAL conferred harmful effects towards these insects besides giving substantial protection to the rice plants.

Materials and Methods

Transformation vectors

Two Ti plasmid based super-binary vectors, containing the selectable marker gene *bar* driven by a CaMV 35S promoter; and the *gna* gene driven by the Phloem specific rice sucrose synthase promoter (RSs1) and *asal* gene driven by CaMV 35S promoter were constructed. Expression cassettes of *bar* (CaMV 35S-*bar*-*nos*) (Rathore *et al.,* 1993), *gna* (Rss1-*gna*-*nos*), and *asal* (CaMV 35S-*asal*-*nos*), were cloned at the multiple cloning site of the intermediate vector pSB11 (Komari and Kubo, 1999), obtained from Japan Tobacco Inc., Japan. The recombinant clones were introduced into *Agrobacterium* strain LBA4404 by triparental mating (Lichtenstein and Draper, 1985), and the resulting co-integrate vectors were designated as pSB111Rss1-*gna*-35S*bar* and pSB111CaMV 35S-*asal*-35S*bar* (Fig. 1a and b).

Genetic transformation studies using pSB111super-binary vectors

The local popular *indica* rice cultivar, namely, Chaitanya (susceptible to major insect pests) was used for genetic transformation experiments using the super-binary vectors pSB111Rss1-*gna*-35S*bar* (Fig. 1a) and pSB111CaMV35S-*asal*-35S*bar* (Fig. 1b). The GNA transgenic lines were developed and

BASTA leaf dip assay

Thirty to forty day old putative transformants were tested along with controls for their tolerance to the herbicide BASTA. The regenerated plants were tested by dipping the apical portion of leaf (7-9 cm) into 0.25% BASTA solution. The leaves were monitored after 72h for signs of damage.

Molecular analysis

The transgenic plants employed in this study were well characterized by Southern and northern blot analysis (Nagadhara *et al.,* 2003; Yarasi *et al.,* 2008; Yarasi *et al.,* 2011). The amount of GNA in the transgenic rice plants was estimated to be 0.1% - 0.3% of total leaf soluble proteins, in comparison with GNA standards on the blots (Nagadhara *et al.,* 2003; Yarasi *et al.,* 2008; Yarasi *et al.,* 2011). And the amount of ASAL in the transgenic rice plants was estimated to be 0.7%-1.49% of total leaf soluble proteins, in comparison with ASAL standards on the blots (Nagadhara *et al.,* 2003; Yarasi *et al.,* 2008; Yarasi *et al.,* 2011).

Insect bioassays

In planta insect bioassays using BPH insects were carried out on homozygous transgenic rice lines and untransformed control plants. All insect bioassays were carried out at the Directorate of Rice Research (DRR) as

described earlier (Nagadhara *et al.,* 2003; Yarasi *et al.,* 2008; Yarasi *et al.,* 2011).

Insect survival assays

Thirty day old homozygous transgenic rice plants of ASAL transgenic line (T49) and GNA transgenic line (OU-1) and untransformed control plants were used to assess insect mortality /survival in no choice method. Early 1st instar nymphs, 20 each, of BPH were independently released on each plant and confined in an insect proof nylan cage in 10 replications. Survival was monitored and observations were recorded on the nymphal survival for every 6 day intervals up to 24 days (Nagadhara *et al.,* 2003; Yarasi *et al.,* 2008; Yarasi *et al.,* 2011). Data were analyzed using the sigma plot software, version 5.0, for windows (SPSS, Richmond, California, USA).

Honeydew (liquid excreta) assay for estimation of feeding ability of insects

The extent of insect feeding was measured by semi-quantitative assay of the honeydew produced (Nagadhara *et al.,* 2003; Yarasi *et al.,* 2008). Whatman No.1 filter paper dipped in a solution of bromocresol green (2mg/ml in ethanol) was used for honeydew estimation. The filter paper was placed at the base of each plant and covered with a plastic cup. On each plant five female adult insects of BPH, pre-starved for 2h, were released separately, and allowed to feed for 24h to 96h. Care was taken not to release gravid adult females. Insects excreta (honeydew) react with the bromocresol green on the filter paper leading to development of blue colored spots. The spots observed on the bromocresol green paper were blue or green in colour or seen as white or transparent spots. The blue colour spots indicate the feeding from phloem since pH is alkaline. The green colour indicates a transition from orange to blue colour formation. The white transparent spots indicate the feeding on the xylem since the water pH is neutral. The area of blue spots developed on the filter paper was measured using the millimeter graph paper and expressed in $1mm^2$ units (Nagadhara *et al.,* 2003; Yarasi *et al.,* 2008; Yarasi *et al.,* 2011). The observations were recorded for every 24h by replacing the new filter paper.

Results and Discussion

Rice is being an important food crop is attacked by more than 100 insect species which cause significant economic loss in various regions. Pest problem increased with the intensification of irrigated rice production, which increases cost of production. Plant hoppers are common rice insect pests in Asian rice production regions. Hopper burn is a non-contagious disease of plants caused by the direct feeding damage of certain leafhoppers and plant hoppers. Hopper burn is caused by a dynamic interaction between complex insect feeding stimuli (termed hopper burn initiation) and complex plant responses (termed the hopper burn cascade). It has been emerged as a potential threat to rice production in tropical Asia. In the current "Post – Green Revolution era," emphasis is given on sustainability and efficiency rather than on further intensification with expensive inputs. In pest management, the challenge is to make natural non-chemical methods collectively more effective. Moreover botanical insecticides are naturally occurring chemicals extracted from plants.

The transgenic rice lines, containing *asal* and *gna* genes, were obtained by genetic transformation using *Agrobacterium* super-binary vectors pSB111Rss1-*gna*-35S*bar* and pSB111CaMV35S-*asal*-35S*bar*. Both the transgenic lines were thoroughly characterized by molecular, genetic and insect bioassays experiments. The presence and

expression of transgenes (*asal* & *gna*) in transgenic rice lines were confirmed through PCR, Southern, Northern, western blot analyses and insect bioassays (Fig. 2) (Yarasi *et al.*, 2008, 2011). Although the constitutive CaMV35S gene promoter, used in many constructs for expression in transgenic plants, is expressed efficiently in phloem tissue, it was felt desirable to identify promoters that would show phloem-specific expression for use in producing rice with BPH resistance. Use of such promoters could give higher levels of expression in the phloem and would minimize exposure of non-target insects and other consumers of the plant material. Plant lectins are considered a complex and heterogeneous group of proteins due to the obvious differences in molecular structure, biochemical properties and carbohydrate-binding specificity.

Effect of GNA and ASAL on the survival of BPH

BPH nymphs fed on homozygous ASAL and GNA transgenic rice plants showed a significant decline in survival from the 9th day onwards (Fig. 3). BPH survival on ASAL transgenic rice plants reduced to a mean of 3.30±1.08 insects /plant and on GNA transgenic rice lines 5.30±0.89 insects/plant compared to a mean of 14.20±1.47 insects/plant on control plants over a 24-day bioassay period (Fig. 3). The BPH nymphal survival on ASAL and GNA transgenic rice lines was reduced by 78.9% and 62.7% respectively, when compared to control plants.

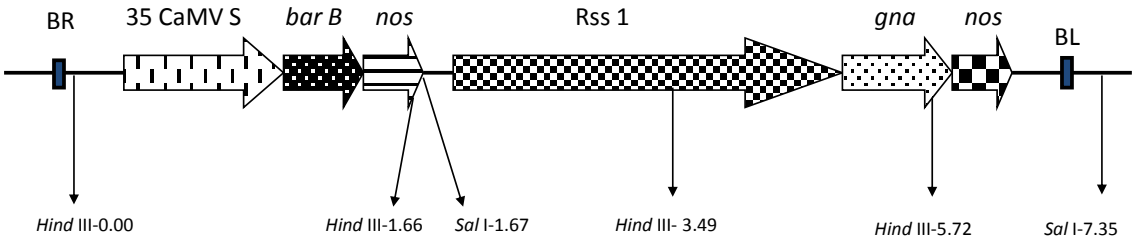

Fig.1a

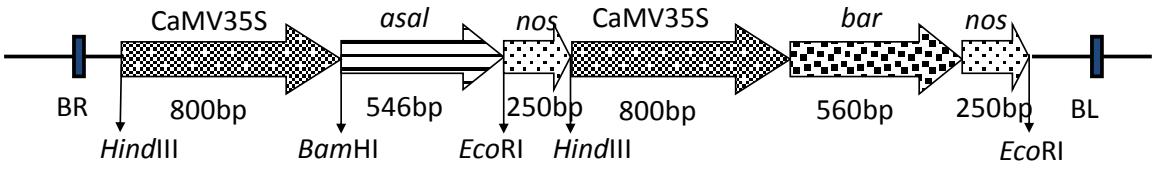

Fig.1b

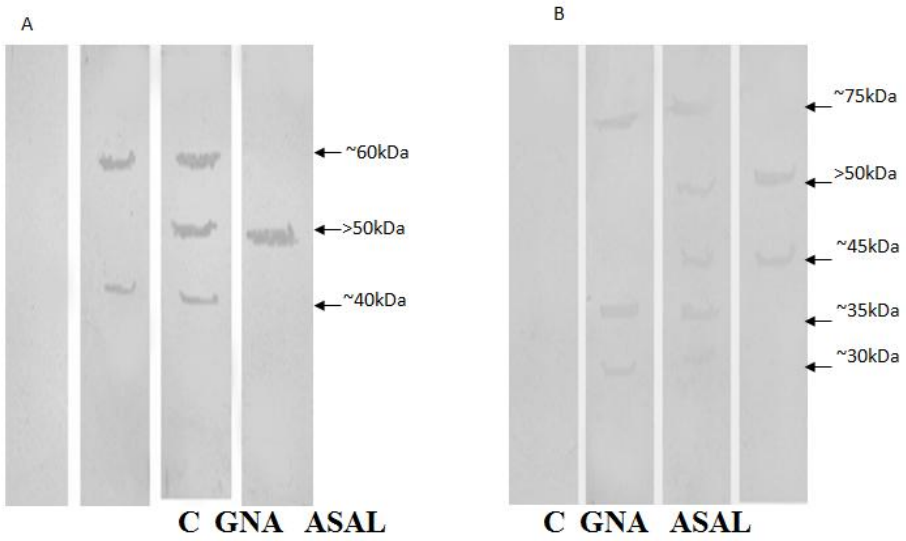

Fig.2 Western blot analysis from insect feeding on showing the ASAL and GNA transgenics

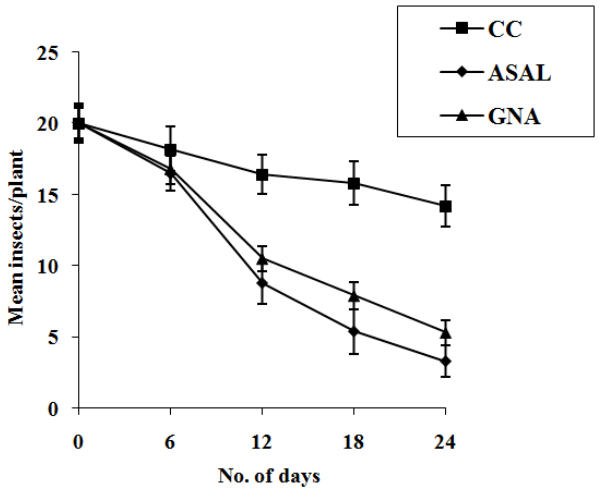

Fig.3 Mean Number of insects survived after feeding on the transgenic plants

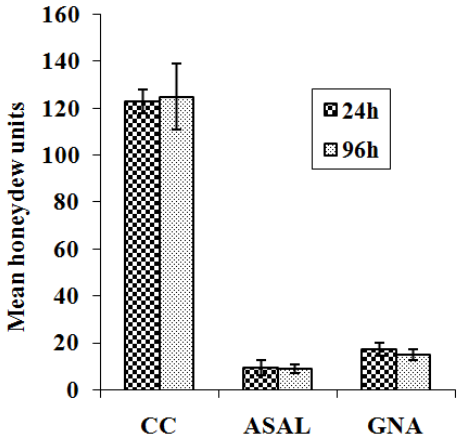

Fig.4 Mean Number of honeydew units after feeding on the transgenic plants

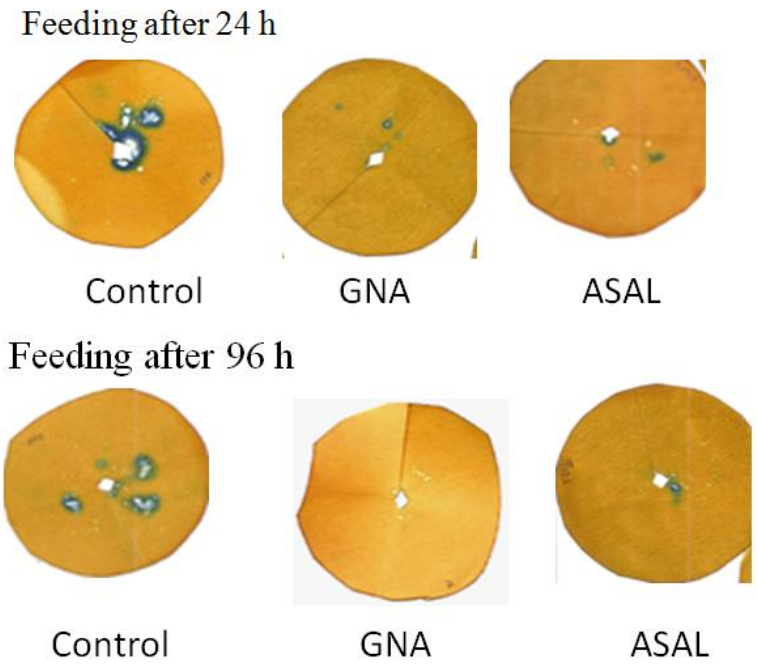

Fig.5 Honeydew assay for BPH after feeding on the transgenic plants

Impact of transgenic rice lines (ASAL and GNA) on the feeding behaviour of BPH insects

Effect of ASAL and GNA on the feeding behaviour of BPH insects was assayed separately by estimating the amount of excreta (honeydew). A mean number of 7.30± 1.10 and 16.10±1.30 honeydew units (blue spots) were excreted by BPH insects fed on ASAL and GNA transgenic rice plants respectively, compared to a mean number of 94±3.50 honeydew units on control plants after 24h of feeding (Fig.4). A mean number of 2.30±0.82 and 1.10 ±0.53 honeydew units (white spots) were excreted by BPH insects fed on ASAL and GNA transgenic rice plants respectively, compared to a mean number of 29±2.30 honeydew units (i.e. white spots indicating the xylem feeding) were excreted on control plants (Fig.4) after 24h of feeding.

A mean number of 6.80±1.29 and 3.20 ±1.28 honeydew units (blue spots) were excreted by BPH insects fed on ASAL and GNA transgenic rice plants respectively, compared to a mean number of 93±12.13 honeydew units (i.e. blue spots indicating the phloem feeding) were excreted on control plants (Fig. 4) after 96h of feeding. A mean number of 2.10±0.78 and 12 ±4.23 honeydew units (white spots) were excreted by BPH insects fed on ASAL and GNA transgenic rice plants respectively, compared to a mean number of 32±2.60 honeydew units (i.e. white spots indicating the xylem feeding) were excreted on control plants (Fig. 4) after 96h of feeding.

After 24 h of feeding on control, ASAL and GNA transgenic rice plants by BPH insects the blue and white colored spots observed on the bromocresol green papers were measured. The blue spots indicate the phloem feeding of the insects and white spots indicate the xylem feeding of the insects. A mean of 9.60±2.30 and 17.20 ±2.70 honeydew units (blue spots) were excreted by BPH insects fed on ASAL and GNA transgenic plants respectively, compared to a mean of 123±5.10 honeydew units on control plants (Fig. 5) after 24h of

feeding, showing a significant reduction of 92.1% and 86.1% in the feeding of BPH insects respectively, on ASAL and GNA transgenic rice plants, compared to control plants. The mean of 8.90± 1.90 and 15.20 ±2.30 honeydew units (blue spots) were excreted by BPH insects fed on ASAL and GNA transgenic rice plants respectively, compared to a mean of 125±14.00 honeydew units on control plants (Fig. 5) after 96h of feeding, exhibiting a significant reduction of 92.8% and 87.8% in the feeding behaviour of BPH insects, on ASAL and GNA transgenic rice plants compared to control plants.

The rice brown planthopper (BPH; *Nilaparvata lugens*) is a serious pest of rice crops throughout Asia, damaging plants both through its feeding behavior and by acting as a virus vector. Like many homopteran pests of crops, it is primarily a phloem feeder, abstracting sap via specially adapted mouthparts. An artificial diet bioassay system for this pest was developed to allow the effects of potentially insecticidal proteins to be assayed. Several lectins and oxidative enzymes were found to be toxic to BPH. BPH in addition to causing direct damage to the plant itself, also act as the vector for stunt viruses. Special attention was focused on homopteran rice pests such as BPH because regular insecticide spraying under intensive farming practices to control these insects has resulted in the loss of natural predators and the selection of pesticide-resistant biotypes allowing pest resurgence. Although BPH-resistant varieties were identified from the germplasm collection, resistance-breaking biotypes have rapidly overcome resistance mechanisms introduced by conventional breeding. As a component of IPM strategies for rice, new resistant varieties are required. Homoptera are sap-sucking insects or phloem-feeders, and so it was considered that in addition to expressing the protein constitutively, specific expression in the phloem would deliver the protein efficiently to the insect while minimizing any potential undesirable accumulation of the protein in other parts of the plant. More importantly, the transgenes in most of the plants were inherited as Mendelian traits.

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How to cite this article:

Bharathi, Y., V.D. Reddy, K.V. Rao and Pasalu, I.C. 2018. Effect of Mannose Specific Lectins *ASAL* and *GNA* on the Feeding Behavior of BPH (*Nilaparvata lugens stal*.). *Int.J.Curr.Microbiol.App.Sci.* 7(09): 1426-1436. doi: https://doi.org/10.20546/ijcmas.2018.709.171