# Dietary Effects of Cotton Tissue Expressing Germin Like Protein on Beet Armyworm (Lepidoptera: Noctuidae) Growth, Survival and Pupation

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# DIETARY EFFECTS OF COTTON TISSUE EXPRESSING GERMIN LIKE PROTEIN ON BEET ARMYWORM (LEPIDOPTERA: NOCTUIDAE) GROWTH, SURVIVAL AND PUPATION

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ABSTRACT

Transgenic cotton lines that ectopically express a cotton germin-like protein (GLP) were screened for resistance/tolerance factors to the beet armyworm (BAW) *Spodoptera exigua* (Hübner) via feeding assays. The number of BAW eggs that successfully hatched was not statistically different at 72 h after infestation for wild-type cotton plants (*Gossypium hirsutum* L. c.v. 'Coker 312') or plants of 4 independent transgenic lines (ABP-A, ABP-B and ABP-C and ABP-D). However, the damage caused by these same larvae at 72 h was higher for 'Coker 312' and line ABP-D when compared to ABP-A, ABP-B and ABP-C transgenic plants. Larval live weights were also significantly higher for Coker 312 and ABP-D at 5, 7, and 14 d when compared to ABP-A, ABP-B and ABP-C. The percentage of larvae that successfully completed pupation was significantly higher for BAW larvae fed 'Coker 312' and ABP-D tissue compared to the other 3 lines. These feeding bioassays show the potential for using cotton germin like protein to improve resistance or tolerance for BAW attacking cotton.

Key Words: bioassays, ectopic expression, germin like protein, plant resistance, *Spodoptera exigua*

RESUMEN

Se evaluaron líneas transgénicas de algodón que expresan ectópicamente una proteína similar a la germina de algodón (GLP) para los factores de resistencia/tolerancia al gusano soldado de la remolacha (GSR) *Spodoptera exigua* (Hübner) a través de ensayos de alimentación. El número de huevos de GSR eclosionados con éxito no fue estadísticamente diferente a las 72 h después de la infestación de plantas de algodón de tipo salvaje (*Gossypium hirsutum* L. cv 'Coker 312') o de plantas de 4 líneas transgénicas independientes (ABP-A,-B y ABP ABP-C y ABP-D). Sin embargo, el daño causado por estas mismas larvas a las 72 h fue mayor para la 'Coker 312' y la línea D-ABP en comparación con las plantas transgénicas ABP-A, B-ABP y ABP-C. El peso de las larvas vivas fue también significativamente mayor para las Coker 312 y D-ABP a los 5, 7, y 14 dias, en comparación con las ABP-A, B-ABP y ABP-C. El porcentaje de larvas que completaron el estadio de pupa con éxito fue significativamente mayor en larvas de GSR alimentadas con la 'Coker 312' y tejido de ABP-D en comparación con las otras 3 líneas. Estos bioensayos de alimentación muestran el potencial para el uso de proteína similar a la germina de algodón para mejorar la resistencia o tolerancia al GSR que ataca algodón.

Palabras Clave: bioensayos, expresión ectópica, proteína como la germina, resistencia en plantas, *Spodoptera exigua*
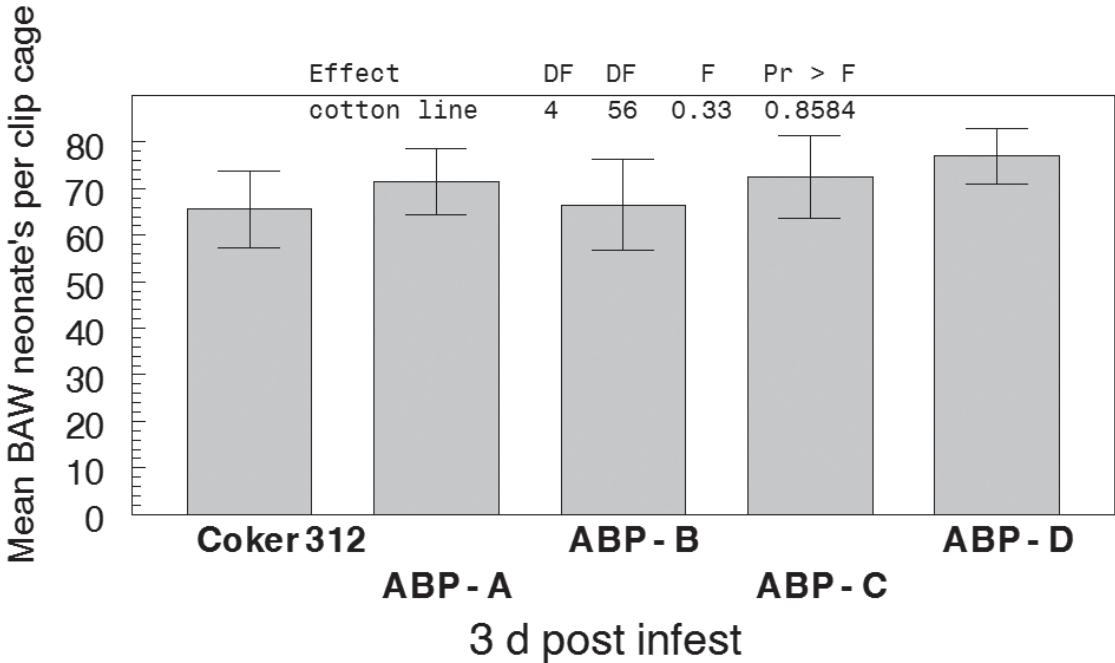
Fig. 1. Mean (± SE) number of beet armyworm neonate larvae that successfully hatched 72 h after attaching egg masses to cotton leaves.

Cotton (*Gossypium hirsutum* L.; Malvales: Malvaceae) production from a global standpoint faces significant challenges from insects, weeds, diseases, soil fertility and limiting rainfall or irrigation. Increases in cotton yields over the last 5 decades have come to fruition with genetic improvements in cotton genotypes through improved fiber production and quality traits. However, one potential improvement in cotton production would be the identification of genes that help alleviate both biotic and abiotic stresses. World-wide production of cotton, as well as many other crops, is significantly affected by environmental stresses including limited available water or rainfall. Some candidate genes in cotton that are involved in a wide range of stresses include transcription factors, protein kinases, ubiquitin ligases, and germin like proteins (GLP) (Abdel-Mageed et al. 2004; Kang et al. 2007; Allen & Aleman 2011). Until these candidate genes are identified and evaluated in greenhouse and field trials, their potential usefulness will never be realized.

The beet armyworm (BAW), *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), is a migratory moth existing in both the eastern and western hemispheres (Smits et al. 1987).

Two important hosts of this insect include cultivated cotton, *Gossypium hirsutum* L., and pigweed *Amaranthus* spp. (Caryophyllales: Amaranthaceae) (Greenberg et al. 2001; Greenberg et al. 2002). Furthermore, the BAW has a preference for drought stressed pigweed and cotton (Showler 2001; Showler 2002). The reproductive potential of BAW is high, with mated females having the capacity to oviposit several egg masses for a total of > 1500 eggs (Rogers & Marti 1997; Tisdale & Sappington 2001). As the eggs hatch, the small larvae are forced to migrate from the oviposition site because the epidermal leaf tissue and fruiting tissue is being consumed. The growth of the larvae and demand for more leaf and fruit tissue is exponential, resulting in plants that are completely defoliated within days. Although BAW infestations are considered secondary, there are crop management efforts that favor severe outbreaks in cotton like the area wide control of the boll weevils where all cotton grown within zones is sprayed with Malathion. Management of BAW using insecticides is difficult, at best, with insecticide resistance developing quickly (Brewer & Trumble 1991). There are well documented observations of multiple endemic outbreaks of
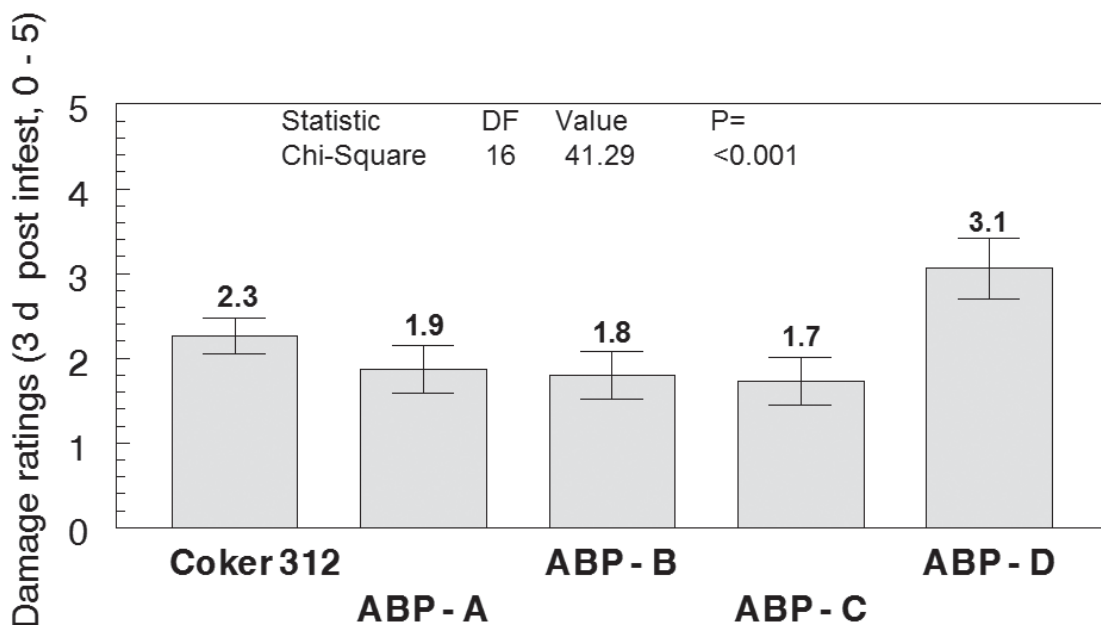
Fig. 2. Mean (± SE) damage ratings based on the amount of cotton tissue consumed by beet armyworm neonate larvae 72 h after feeding Coker 312 and 4 transgene derivatives developed for abiotic stress. Damage ratings were based on a 0 to 5 scale where 0 = no damage, 1 = 1-20% damage, 2 = 20-40% damage, 3 = 40-60% damage, 4 = 60-80% damage, 5 = 80-100%.

BAW within the United States (Smith 1994; Burris et al. 1994; Summy et al. 1996) and the repeated use of most insecticides results in mortality of the generalist predators and parasites that keep BAW populations in check (Eveleens et. al 1973; Ruberson et al. 1994; Summy et al.1996). More recent advances in BAW control have been realized with the incorporation of Bollgard II® and WideStrike® technologies into commercially available cotton varieties (Adamczyk et al. 2008; Siebert et al. 2009).

The cotton GLP1 shares strong homology with the *Arabidopsis* GLP3 as well as the *Prunus persica* auxin-binding proteins (ABPs). Biochemical analysis revealed that *GhGLP1* does not possess enzymatic activities usually associated with GLPs, such as oxalate oxidase and superoxide dismutase (Kim & Tripplett 2004). To establish a biological function of *GhGLP1*, several independent transgenic cotton plants that over-express *GhGLP1* were developed. During the process of developing these plants, we observed that most of the transgenic lines show some tolerance to common greenhouse insects. Given that the role of germin-like proteins in plant-insect interaction is well established (Ramputh et al. 2002; Lou & Bald-

win 2006) we sought to evaluate the response of these transgenic cotton lines to BAW attack. In this experiment we used feeding bioassays to determine the effects of the ectopic expression of *GhGLP1* (Kim & Triplett 2004) on the growth and development of BAW. These cotton lines constitutively express the cotton germin like protein *GhGLP1* under the control of the CaMV 35S promoter. Line ABP-A, ABP-B, and ABP-C are high expressors of the transgene (6 to 7 fold increase relative to wild-type plants) while line ABP-D shows a low level of expression similar to the wild-type Coker 312 (Data not shown).

MATERIALS AND METHODS

Feeding Bioassays

The 4 independent transgenic lines were used in feeding, damage, growth and developmental assays of the BAW. The 4 lines were developed at the Department of Biological Sciences, Texas Tech University, Lubbock, Texas where they were sent as seed to the USDA-ARS Subtropical Research Unit, Weslaco, Texas where they
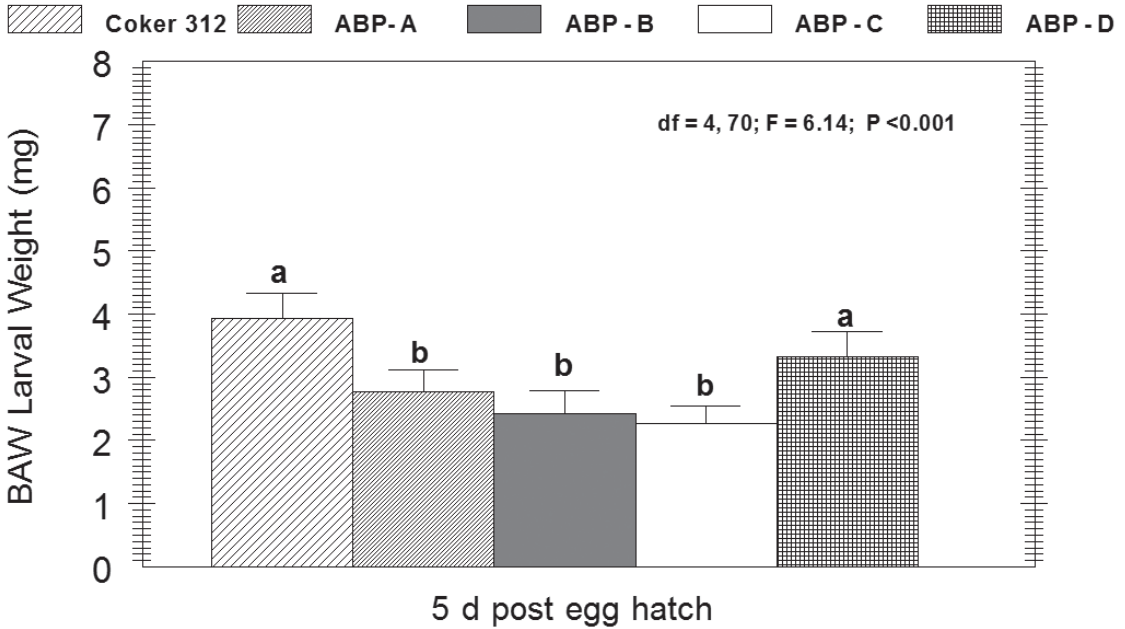
Fig. 3. Mean (± SE) live weights of beet armyworm larvae that survived from feeding on cotton host plant tissue at 5 d post egg hatch. Different letters above the means indicate a significant difference between cotton lines (*P* < 0.05).

were evaluated in BAW feeding bioassays. Cotton seeds labeled (ABP line A, ABP line B, APB line C, and ABP line D) and the parent cv 'Coker 12' were planted in 11.2 L pots containing a mixture of 1:3 potting soil (Sunshine mix, Bellevue, Washington) to field soil. The cotton plants were maintained in the greenhouse by watering every 10 - 14 d and fertilizing with 5% solution (Peter's fertilizer, 20-20-20, N-P-K, Scott's Sierra Horticultural Products Marysville, Ohio) every other time they were watered. Study plants were 12 nodes above the cotyledon leaves (just beginning to flower, 65 d after planting) when bioassays were initiated. BAW egg masses produced by the USDA-ARS Subtropical Research Unit, Weslaco, Texas insectary were attached to the second or third fully expanded leaf from the terminal. The egg masses originated from paper toweling inside the BAW rearing cage, so the toweling was dissected into small pieces (i.e., 3-4 cm$^2$) to contain an estimated 60-70 eggs. Counting the eggs in each egg mass was not possible, but efforts were made to use approximately the same number of eggs. There were 15 egg masses (replicates) attached to the 4 different ABP lines and the non-transgenic 'Coker 312', which was considered a control (see Fig. 1). The egg masses were then covered with clip cages to prevent the neonates

from escaping after hatching. After 72 h, the infested leaves with the clip-cage still attached, were collected in separate sealable bags and taken into the laboratory where the number of live neonate larvae were counted, and a damage rating (0 = no damage, 1 = 1-20% damage, 2 = 20-40% damage, 3 = 40-60% damage, 4 = 60-80% damage, 5 = 80-100%) was assessed to each leaf that estimated the amount of leaf surface that was consumed (see Armstrong et al. 2011). Mortality and larval weights (Mettler-Toledo AT261 DeltaRange®, Columbus, Ohio) were assessed at 5, 7 and 14 d for 15 larvae from each clip cage (replication) by transferring them to a fully expanded cotton leaf maintained in a 15 cm diameter Petri dishes lined with Whatman® (Buffalo, New York) filter paper. Cotton leaf tissue was changed every 48 h from the original plants maintained in the greenhouse. Petri dishes containing beet armyworms were held in an environmental growth chamber at 28.5 °C, 65% RH and at 14:10 h L:D.

Statistics

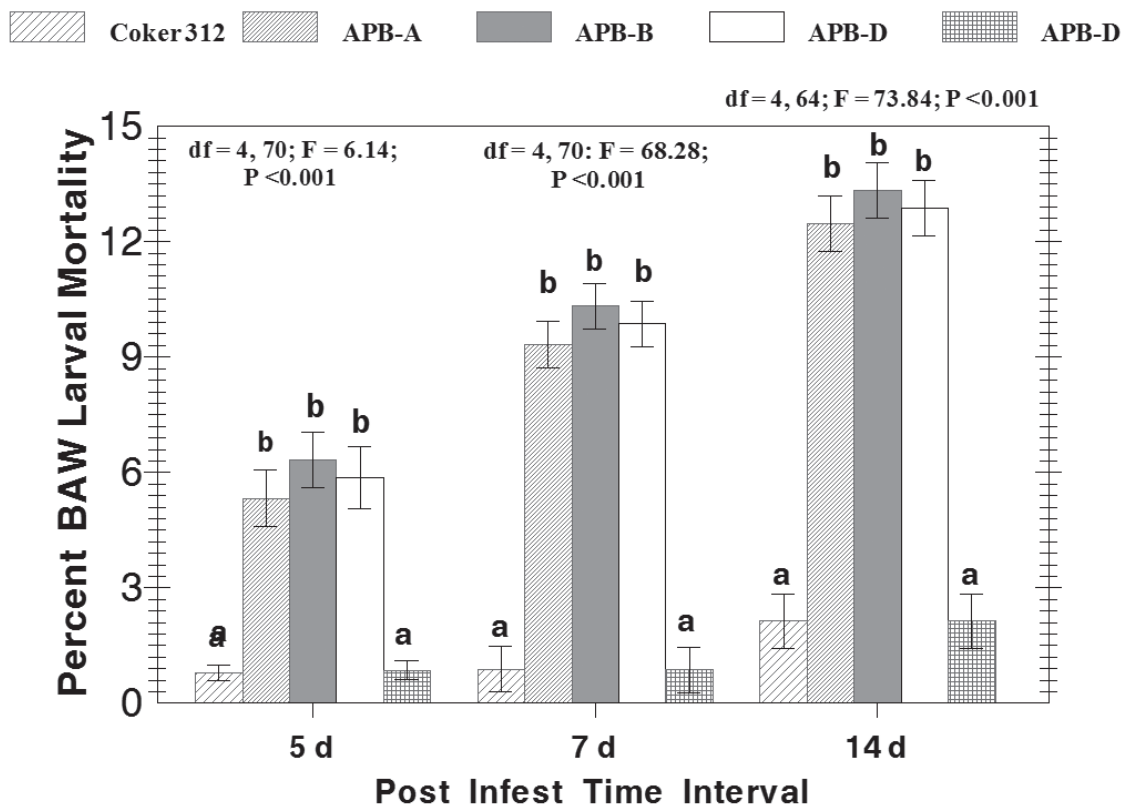The number of BAW larvae that were successful in eclosion at 72 h**,** larval mortality, and

Fig. 4. Mean (± SE) percentage mortality of beet armyworm that fed on cotton plant tissue at 5, 7 and 14 d post egg hatch. Different letters above the means indicate a significant difference between cotton lines ($P < 0.05$).

larval live weights at 5, 7 and 14 d were analyzed with the GLIMMIX procedure of Mixed Model Analysis; Degrees of freedom were calculated using the Satterthwaite method (SAS, 2008, version 9.2, SAS Institute Inc., Cary, NC). Means were separated with LS MEANS ($\alpha = 0.05$). Because of the subjective nature of damage ratings, data were analyzed with the PROC FREQ procedure and compared using the Chi-Square test ($\alpha = 0.05$).

### RESULTS AND DISCUSSION

The number of neonate BAW that successfully hatched at 72 h from each group of egg clusters across all cotton lines was not statistically different (Fig. 1). Only 3 larvae out of the hundreds tested were dead at 72 h, therefore mortality was not a factor and there were no immediate deleterious effects from the con-

sumption of cotton tissue. However, the damage ratings conducted at 72 h were statistically different (df = 16, $Chi^2 = 41.3$, $P = <0.001$, Fig. 2). The damage ratings for ABP-D was higher than the parent 'Coker 312', but feeding injury for ABP-A, ABP-B, and ABP-C were significantly lower than 'Coker 312'.

Live weights for larvae at 5 d were somewhat reflective of the damage ratings in that the Coker 312 weights were the highest and not significantly different from the ABP-D line. Live weights of larvae at 5 d from the ABP-A, ABP-B and ABP-C were significantly less than Coker and ABP-D, but not significant from each other (df = 4, 70: $F = 6.1$; $P = < 0.001$, Fig. 3). The live weights were reversed when compared to the consumption of tissue indicated by damage rating in that the ABP-D line had a higher damage rating, but live weights were numerically higher for the 'Coker 312' parent. The reason that the ABP-D line had a higher damage rating but lower live larval weights compared to
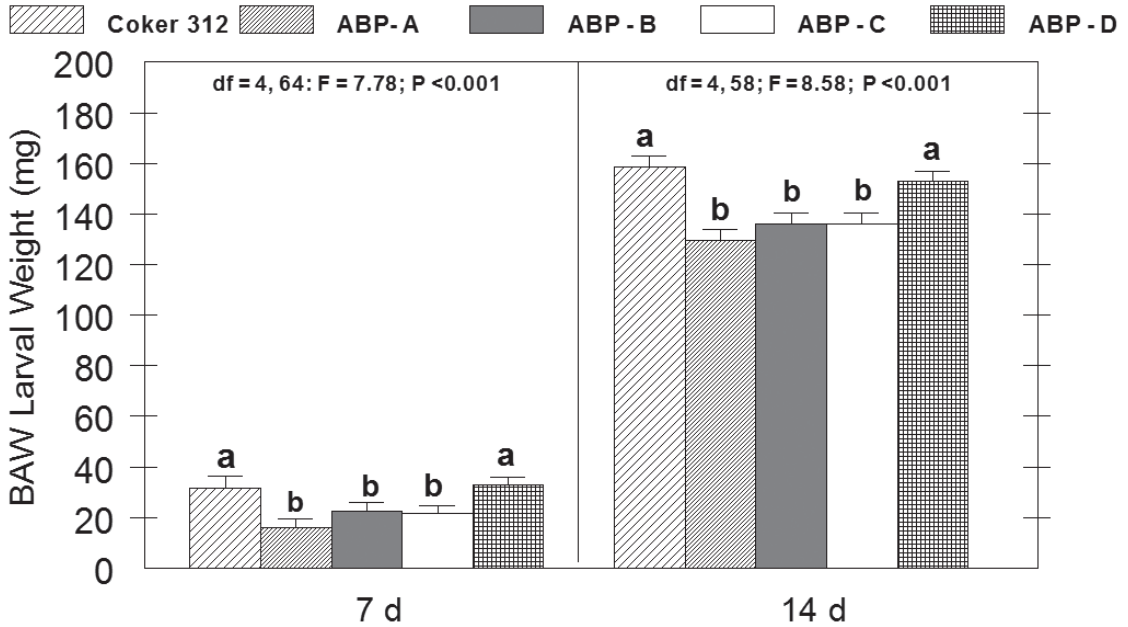
Fig. 5. Mean (± SE) live weights for beet armyworm larvae fed cotton plant tissue at 7 and 14 d post egg hatch. Different letters above the means within an observation period, indicate a significant difference between cotton lines (*P* < 0.05).

'Coker 312' could be a result of having a higher number of successful larvae from the ABP-D line, although this was not statistically apparent at the initiation of the feeding assay.

The observations of larval mortality indicated a clear and significant pattern of separation among the cotton lines tested. Mortality at 5 d was significantly lower for the 'Coker 312' and APB-D when compared to the remaining ABP lines on the order of 4-5% higher (Fig. 4). At 7 d, mortality of larvae feeding on ABP-D was < 1%, while mortality in the remaining 3 lines (ABP-A, ABP-B, ABP-C) ranged from 9.5 to 11.0 %. At 14 d, mortality of larvae on the Coker parent and ABP-D was approximately 3%, while the remaining 3 lines were > 12% (Fig. 4). Also the live weights of larvae on the Coker parent and ABP-D were slightly larger than on the other lines (Fig. 5).

The percentage of BAW larvae that successfully pupated was almost identical for the ABP-A, ABP-B and ABP-C lines averaging just over 70% (Fig. 6). However a higher (df = 4, 70, *F* = 14.6, *P* < 0.001) percentage of BAW successfully pupated when fed the 'Coker 312' at 90% success followed by 89% for ABP-D.

Germin and germin-like proteins (GLPs) are ubiquitous plant proteins that belong to large multi-genes families. Different members of GLP are temporally and spatially expressed and many are responsive to biotic and abiotic signals (Dunwell et al. 2000; Lane 2002). Several reports implicated GLPs in plant-insect in-
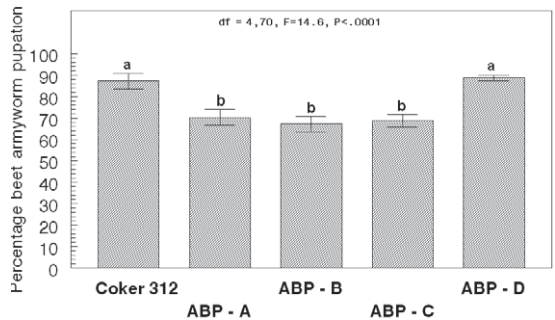


Fig. 6. Mean (± SE) percentage of beet armyworm neonate larvae that successfully pupated after being fed cotton tissue. Different letters above each bar indicate a significant difference between cotton lines, (*P* < 0.05).

teraction. For example, Ramputh et al. (2002) reported that expression of wheat germin gene in corn confers resistance to the European corn borer *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). Furthermore, Lou & Baldwin (2006) show that silencing a GLP gene in *Nicotiana attenuata* Torr. ex S.Watson (Solanales: Solanaceae) improves performance and preference of 2 native herbivores. Although the exact mechanism by which GLPs improve tolerance to herbivores is not well understood, authors of several studies speculate that it might involve modifications of plant cell wall, up regulation of pathogen resistance genes and $H_2O_2$ burst.

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