Submitted Papers

Microscopic examination of *Lygus lineolaris* (Hemiptera: Miridae) feeding injury to different growth stages of navy beans

T. Nagalingam and N.J. Holliday

Department of Entomology, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2
Corresponding author e-mail: Neil_Holliday@umanitoba.ca

Abstract — Light microscopy was used to study feeding injury to navy beans, *Phaseolus vulgaris* L. (Fabaceae), by fifth instar nymphs and unmated newly-moulted adults of *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae). Insects were caged on individual racemes of potted plants at different growth stages. From flowering to early pod set, abortion of pods, flowers or buds was the most common response to feeding injury; this type of injury did not occur at later growth stages. During the seed development growth stage, feeding resulted in exterior diffuse discoloured patches on seed pods and associated interior necrosis of the vascular tissues supplying seeds. At harvest, many of the seeds were shrivelled when racemes were fed upon during the seed development growth stage. Direct seed injury, involving penetration of the testa and loss of cotyledon tissue, was occasionally observed during the seed development growth stage, but was the most frequent injury at the developed seed stage. Harvested seeds that were directly injured exhibited crater-like surface pits fringed by brown pigmented areas. There were no observable differences in the type of injury caused by fifth instar nymphs and the adults at any of the growth stages.

Introduction

Mirid bugs in the genus *Lygus* Hahn are major crop pests worldwide (Tingey and Pillemer 1977). In North America, *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) feeds on plants of more than 385 species (Young 1986), among them *Phaseolus vulgaris* L. (Fabaceae), a species that includes green beans with edible pods and dry edible beans (also known as field beans). *Lygus* feed on meristematic tissues and developing reproductive organs of plants (Strong 1970) by inserting their piercing-sucking mouth parts into the tissues and mechanically or enzymatically destroying cell walls and liberating cell contents (Hori 2000; Backus *et al*. 2007). The cell contents are then digested and consumed through cycles of alternating flushing with saliva and ingestion of the resulting fluids (Strong 1970, Miles 1972; Backus *et al*. 2007). Enzymes in *Lygus* saliva include polygalacturonases, α-amylases, proteases and phenoloxidases (Strong and Kruitwagen 1968; Celorio-Mancera *et al*. 2008; Cooper *et al*. 2013).

Injury by *Lygus*, mostly from feeding on reproductive plant tissues, has been studied macroscopically in apple (Michaud *et al*. 1990), buckwheat (Mostafa 2007) and sunflower (Charlet 2003). There have been light microscopic studies of *Lygus* injury to cotton (King and Cook 1932, Williams and Tugwell 2000), sugar beet and rape (Hori 1971), pumpkin (Hori *et al*. 1987), guayule (Addicott and Romney 1950) and wheat (Wise *et al*. 2000), and light and scanning electron microscopy studies of *L. lineolari*s injury to canola (Butts and Lamb 1990), strawberry (Handley and Pollard 1993a, 1993b), and grape vine (Fleury *et al*. 2006). Macroscopic studies of *Lygus* injury to beans of the genus *Phaseolus* (L. (Fabaceae)) have been carried out on lima beans, *P. lunatus* L. (Baker *et al*. 1946; Bushing and Burton 1974; Elmore 1955), and green beans, *P. vulgaris* L. (Khattat and Stewart 1975). There have been no microscopic studies of *Lygus* injury to *Phaseolus*, and no studies of injury to the field bean types of *P. vulgaris*.

The reproductive organs of *P. vulgaris* are compound racemes, arising in the axil of a trifoliate leaf; three flower buds are located in the leaf axil and at each of two to five nodes along the peduncle of the raceme (Adams 1967; Mauk *et al.* 1984; Sage and Webster 1987). Flowering and pod development occur earlier at the base of the raceme (Sage and Webster 1987). In healthy plants, abortion of buds, flowers or young pods, results in far fewer developed pods than the initial number of buds (Tamas *et al*. 1979; Sage and Webster 1987). Pods are modified leaves, and the two halves of the pod are linked by dorsal and ventral sutures, with the dorsal suture corresponding to the leaf midrib (Woodcock 1935). The vascular supply to developing seeds is the placenta, consisting of two vascular bundles running the length of the pod near the ventral suture; each developing seed is connected to the placenta by the vascular tissue of the funiculus (Woodcock 1935). In healthy pods, considerable numbers of seeds fail to develop, and remain flattened and shrivelled (Harris 1915).

Field beans, including navy beans, are an important crop in Manitoba (Manitoba Pulse and Soybean Growers 2015) and there has been concern about the effect of *Lygus* on this crop (Gavloski 2001–2010). It has been reported that 5–20% of field bean seed can be damaged by *Lygus* (Agriculture and Agri-Food Canada 2005), and processors in Manitoba diagnose blemishes on seeds as insect damage. In Manitoba, >90% of mirids in field bean crops belong to the genus *Lygus* and most of these are *L. lineolaris* (Nagalingam and Holliday 2015). The lack of information about how *L. lineolaris* affects reproductive growth and the quality of seed of field beans impairs diagnosis of *Lygus* injury in the field and accurate assessment of the economic significance of these insects in the crop. Therefore, in this study, we used reflective light microscopy to investigate the feeding injury inflicted by fifth instar nymphs and adults of *L. lineolaris* on reproductive organs of navy beans, *P. vulgaris*, at three different growth stages.

Materials and Methods

Studies were carried out in 2009–2010. Determinate bush-type navy beans of the cultivar 'Envoy' were grown from seed in pots (21 cm diameter, 21 cm height) in a controlled environment room at 23 °C, 70% RH and 16:8 h L:D photoperiod. Pots were filled with horticultural potting mix (Sunshine LA4, Sungro Horticulture Distribution, Inc., Agawam, Massachusetts, United States of America). Two seeds were planted in each pot and, when the first trifoliate leaf opened, plants were thinned to one per pot. Seeds had not received treatment with inoculant or pesticides. Pots were watered every second day, and every two weeks received 0.625 g of NPK 20-20-20 fertilizer in 250 ml of water.

A laboratory colony of *L. lineolaris* was established from over-wintered adults collected each spring from alfalfa fields near Carman, Manitoba. The colony was maintained in a controlled environment chamber at 21 °C, 70% RH and 16:8 h L:D on store-bought broccoli and green beans. First to third instar nymphs were maintained on broccoli pieces in 60 mm diameter polystyrene Petri dishes. Older nymphs and adults were kept in perforated plastic tubs (12 cm diameter, 15 cm height; Bug Tub® (Royal Oak Point NW, Calgary, Alberta, Canada) and provided with green bean (*P. vulgaris*) pods as food and oviposition substrate. Adults were confined with bean pods for 5 days, and then the pods were transferred to 145 mm diameter polystyrene Petri dishes. Thereafter, for one week, the Petri dishes containing the pods were inspected every second day and nymphs that hatched were transferred with a paint brush to the small Petri dishes used for rearing early instar nymphs. The colony was re-established each year to ensure colony vigour.

Injury treatments were made by caging reproductive structures of potted navy bean plants growing in the controlled environment room. Treatments lasted for 5 days during which cages contained either *L. lineolaris* fifth instar nymphs, adults or no insects (controls). Cages (Fig. 1) were fabric sleeve (12 cm diameter, 30 cm long) of 40 thread/cm mesh tergal netting (Fabricland, Winnipeg, Manitoba, Canada) on a wire ring frame. Each cage

was supported by attaching it to a wire tomato support frame, the base of which was pushed into the soil of the pot. A single raceme and its subtending trifoliate leaf were inserted into the sleeve cage and the proximal end of the sleeve was secured around the plant stem with a twist tie. The distal end was tightly secured with an elastic band. Both twist ties and elastic bands were covered with adhesive tape to prevent them being dislodged. Insects introduced into infested cages were either fifth instar nymphs within 1 day of moulting from the previous instar, or adults that had completed their final moult within the previous 1–2 days. To avoid injury from oviposition or feeding by young nymphs during the five-day exposure, adults were isolated from the colony as fifth instar nymphs and not allowed to mate; sex of adults was not determined. Injury treatments were made at three plant growth stages (Brown *et al.* 2017), stages present when *L.*

Figure 1. Sleeve cage enclosing raceme of a potted navy bean plant.

lineolaris occurs in navy bean fields in Manitoba (Nagalingam and Holliday 2015), and the number of insects used at each growth stage was chosen to produce injury but not complete destruction of caged plant parts. At growth stages R2–R3 (flowering to early pod set), one nymph or one adult *L. lineolaris* was introduced into each infested cage; there were five replicates of each treatment and of the control. At R4–R5 (mid pod set to early seed fill) three nymphs or three adults were introduced; the treatments and control were replicated six times. At R6–R7 (mid seed fill to early pod maturity) five nymphs or five adults were introduced, and there were three replicates of each treatment and the control.

After the five-day exposure, the insects and cage were removed, and the raceme was detached from the plant and examined under a Leica MS5 stereomicroscope (magnification range 12.6 to 80). Injured plant parts were compared with uninjured parts from control cages, and representative injuries were photographed using a Nikon D5200 digital camera attached to an Olympus SZX16 light microscope. In addition to examination of racemes immediately after exposure to insects, some plants were grown until the stage of commercial seed harvest, and seeds from the previously-caged racemes were examined microscopically as described above.

Results

The use of unmated adults to prevent oviposition injury was successful as there were no eggs or young nymphs seen when sleeve cages were dismantled. Only feeding injuries were evident at the end of the five-day treatment period.

In growth stages R2–R3, buds, flowers and small pods are present (Brown *et al.* 2017). At the end of the five-day exposure, injuries to pods caused by feeding of either adults and nymphs were characterized by brown spots, about 1 mm in diameter, centred at the feeding puncture (Fig. 2A). In some cases, the exterior surface of injured pods was irregular because of swelling at feeding sites (Fig. 2B). In other cases, “split lesions” (Painter 1930) in the epidermis occurred at feeding sites (Figs. 2C–E). Split lesions on the peduncle were found mainly in internode regions but usually close to a node, and often resulted in constriction or breakage (Fig. 2D). On pods, split lesions were elongated areas of torn epidermal tissue (Fig. 2E). The most frequent response to feeding injury during the R2–R3 growth stages was abortion of buds, flowers, and pods (Fig. 2F). This abortion injury was readily distinguishable from stem breakage at a feeding site. Abortions occurred in uninfested controls, but were more frequent when racemes were caged with *Lygus*. *Lygus*-induced abortion of pods was associated with brown necrotic lesions inside the pod (Fig 2G). Injuries caused by feeding by nymphs (Figs. 2A–C, G) showed no observable differences from those resulting from adult feeding (Figs. 2D–F).

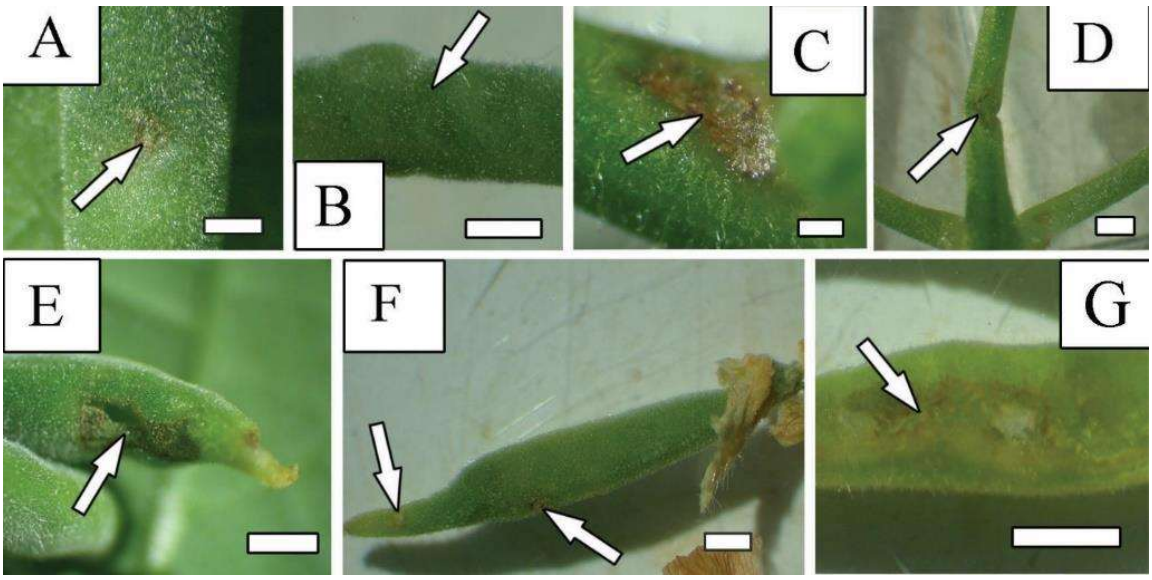

Figure 2. Injuries caused by *Lygus lineolaris* during feeding on the R2–R3 growth stages of navy beans; scale bars = 1 mm. (A) Necrotic spot (arrow) on pod surface at the site of nymphal feeding; (B) pod showing swellings (arrow) at sites of nymphal feeding; (C) discoloured area and split lesion (arrow) on peduncle of the raceme resulting from nymphal feeding; (D) split lesion (arrow) on peduncle following adult feeding; (E) split lesion (arrow) on pod following feeding by an adult; (F) pod aborted following feeding by an adult showing location of feeding sites (arrows); (G) aborted bean pod sectioned longitudinally to show inner discolouration following nymphal feeding.

In the R4–R5 growth stages, pods contained seeds that ranged in stage from just initiated to fully developed. At the end of the five-day exposure, external pod injury in the R4–R5 growth stage took the form of indistinct brownish areas, which were mainly near the ventral suture (Fig. 3A). Stylet entry points near the ventral suture were associated with brown necrotic lesions in the placental region of the seed pod (Fig. 3B); frequently this necrosis also involved the funiculus supplying the developing seed (Fig. 3C). Neither adults nor nymphs showed any preference for specific seed positions within the pod when funiculus injuries were evaluated immediately after exposure to insects.

Direct injury to developing seeds was also observed in the R4–R5 stages, and affected 3% of developing seeds exposed to feeding by three nymphs per raceme, and 7% of seeds

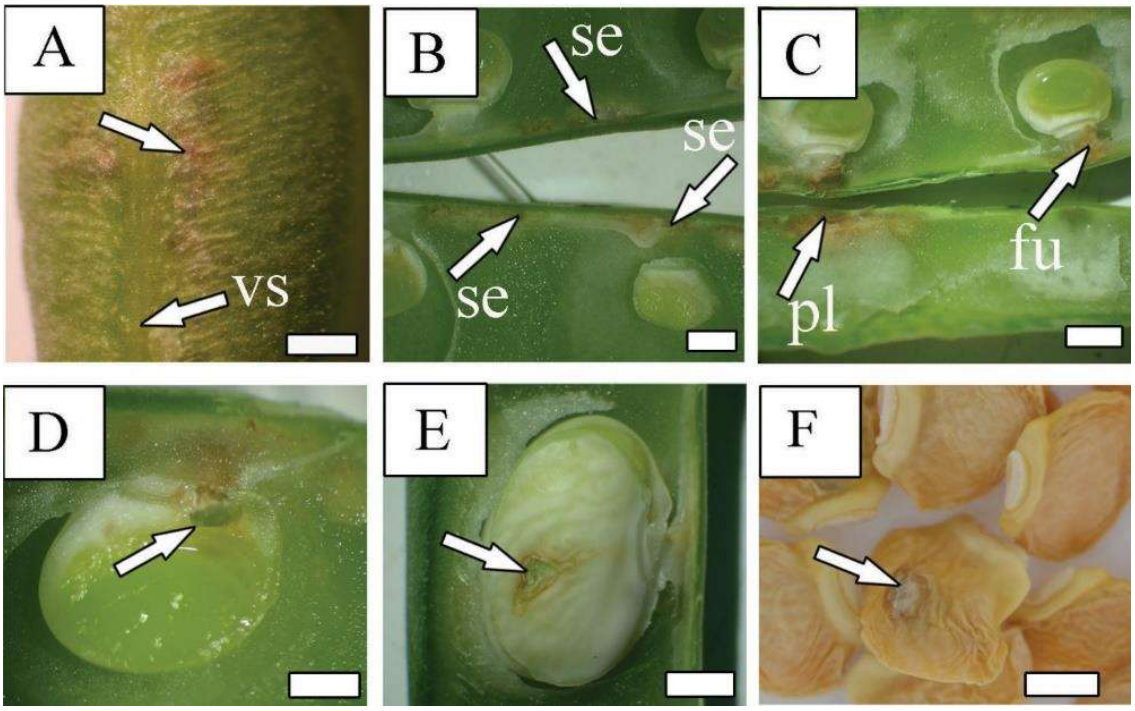

Figure 3. Injuries caused by *Lygus lineolaris* during feeding on the R4–R5 growth stages of navy beans; scale bars = 1 mm. (A) External discolouration (arrow) near the ventral suture (vs) following nymphal feeding; (B) longitudinal section of a pod showing necrosis of the placental region (brown discolouration) and stylet entry points (se) following nymphal feeding; (C) necrosis of the placental region (pl) and funiculus (fu) regions following adult feeding; (D) direct seed injury (arrow) from adult feeding, that penetrated through the testa into the cotyledon of a young developing seed; (E) direct seed injury (arrow) to an older developing seed following nymphal feeding; (F) shrivelled seeds at harvest maturity, one of which has a scar (arrow) from direct seed injury, following feeding injury by nymphs at the R4–R5 stage.

exposed to three adults per raceme. In direct seed injury, the testa was penetrated and a cavity was evident in the underlying cotyledon (Fig. 3D). In the early stages of seed development, there was little change in pigmentation, but in older seeds, the testa bordering the injury became brown (Fig. 3E). Unlike earlier growth stages, no pod abortion resulted from feeding in the R4–R5 stage. Like earlier stages, the nature of injury in response to feeding by nymphs (Figs. 3A, B, E) and adults (Figs. 3C, D) did not differ.

When seeds from the R4–R5 treatments were examined at the time of seed harvest, many of them were shrivelled (Fig. 3F). The majority of shrivelled seeds showed no signs of direct injury, but some shrivelled seeds did have scars indicating that they had been

directly injured. Exposure to nymphs resulted in 94% of seeds being shrivelled at harvest, compared with 70% of seeds in the adult treatment and 37% in controls.

At the R6–R7 stages, bean pods contain fully developed and filling seeds (Brown *et al.* 2017). At the end of the five-day exposure, most injury at these stages was direct injury to the seed, and this did not appear to be fundamentally different from the seed injury in the R4–R5 stage. Exposure to five nymphs per raceme resulted in 37% of seeds being directly injured, whereas 26% of seeds exhibited direct injury when exposed to five adults per raceme. Injury at the R6–R7 stages was more visible because, in more mature seeds, there was greater pigmentation of the testa bordering the injury site (Fig. 4A). The injury was not localized on any particular part of the seed, and more than one injury on a seed was frequently observed. At the site of feeding, the testa was perforated and there was a cavity in the tissues of the cotyledon (Fig. 4B). In most cases, stylets were inserted through the pod wall and the feeding site on the seed was close to the pod penetration point (Fig. 4C); pod penetration points included the dorsal and ventral sutures and sides of the pod. When seeds injured during the R6–R7 growth stages were examined at the normal time of harvest, lesions were crater-like pits and frequently had surrounding concentric raised brown ridges (Fig. 4D), No pod abortion was observed at the R6–R7 growth stages, and no differences were observed between the type of injury from nymphs (Fig. 4B, C, D) and adults (Fig. 4A).

Discussion

The cultivar 'Envoy' was used in this study because the uniformly white seed coat facilitates detection of injury to the seed: the cultivar is the check cultivar with which the performance of other cultivars is compared in Manitoba variety trials. We chose the five-day duration for feeding injury treatments to provide the maximum duration of exposure to a specific insect stage, without transition to a following stage. At the end of the injury treatment at the temperatures of this study, the fifth instar nymphs would be almost ready to moult to adults, and female *L. lineolaris* would be about to begin oviposition (Bariola 1969; Ugine 2012). It is unwise to assume that our results apply to all ages of nymphs and adult *L. lineolaris* as, in *Lygus hesperus* (Knight), feeding behaviour is dependent on nymphal instar (Cooper and Spurgeon 2013) and reproductive status of adults (Cooper and Spurgeon 2011).

In this study, brown discoloured lesions were found both externally and internally in navy bean reproductive organs, regardless of growth stage. The exterior 1 mm diameter brown spots we observed appeared to surround individual stylet penetration points, whereas diffuse brown areas on the surface of pods, were probably the result of multiple low-volume injections of saliva, as described for *L. hesperus* (Backus *et al*. 2007). Brown

colouration was also evident at interior feeding locations, including the sites of placental and funiculus injury. Hori (2000) suggested that oxidation of phenols by phenoloxidases

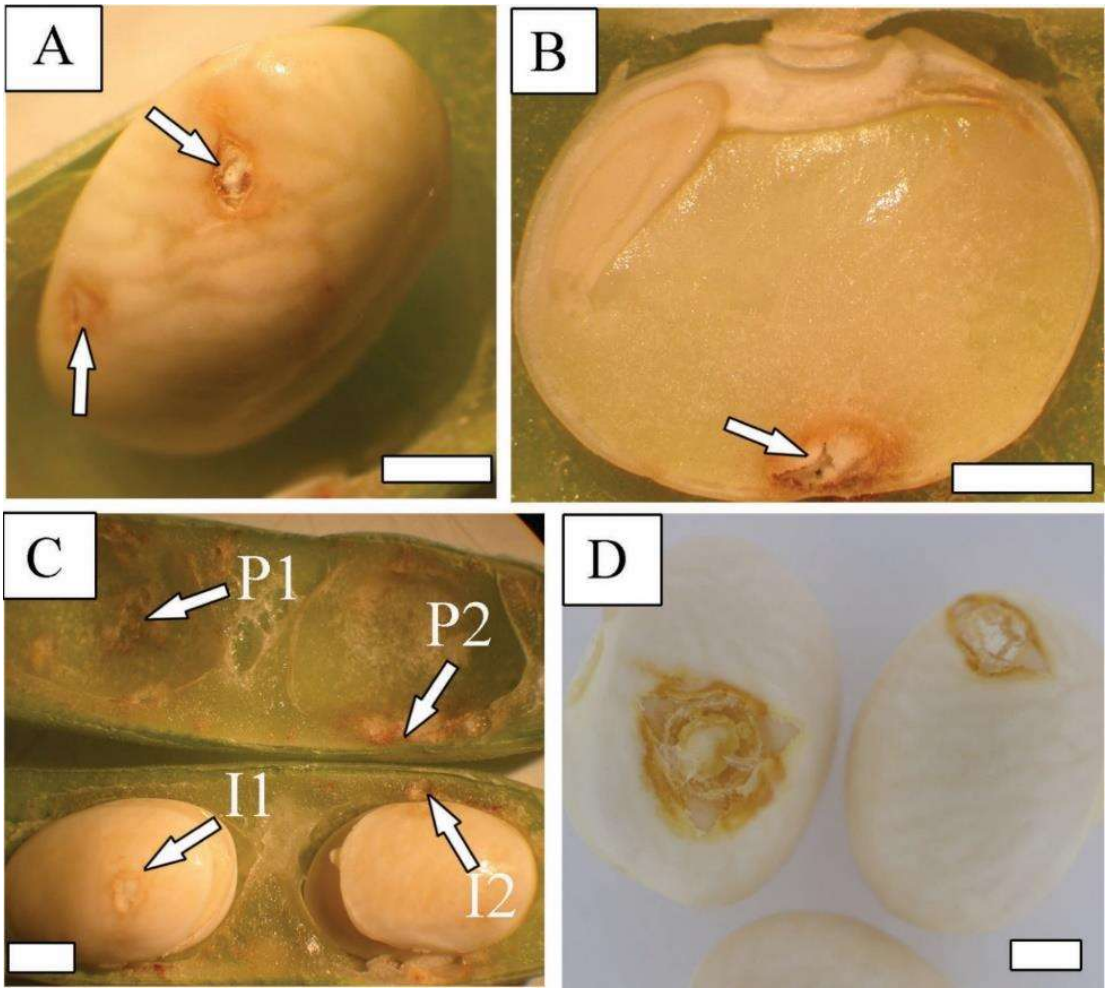

Figure 4. Injuries caused by *Lygus lineolaris* during feeding on the R6–R7 growth stages of navy beans; scale bars = 1mm. (A) Direct injury to seed (arrows) following adult feeding; (B) longitudinal section of a seed showing injury to cotyledon (arrow) following nymphal feeding; (C) corresponding halves of pod joined at the dorsal suture, showing pod penetrations points (P1 and P2) and corresponding seed injury sites (I1 and I2) following nymphal feeding; (D) seed pits on mature harvested seeds following nymphal feeding at the R6–R7 growth stages.

is responsible for the brown-pigmentation following feeding by Heteroptera. In plants, activation of the phenol-phenoloxidase system is a general response to wounding, and so

the brown pigmentation could be the plant's response to cellular destruction by the stylets or saliva of *Lygus* (Hori 2000), or be the result of the phenoloxidases that are minor components of *Lygus* saliva (Cooper *et al.* 2013).

Tissue swelling and split lesions resulted from *Lygus* feeding in the R2–R3 stage. Split lesions on stems are characteristic injuries from *Lygus* feeding on cotton (King and Cook 1932), poplar (Sapio *et al.* 1982) and Douglas-fir (Schowalter *et al.* 1986). Swellings are the result of cell hypertrophy, and interior swelling may strain the epidermis sufficiently to cause it to split (Painter 1930; King and Cook 1932). The hypertrophy probably results from disruption of the plant's hormonal system by *Lygus* feeding (Tingey and Pillemer 1977; Hori 2000), as there is no evidence that *Lygus* saliva contains plant hormones (Strong 1970).

Abortion of reproductive structures in response to *Lygus* feeding has been reported in several leguminous crops including alfalfa (Sorensen 1939), soybeans (Broersma and Luckmann 1970), Lima beans (Baker *et al.* 1946), snap beans (Fisher *et al.* 1946), and green beans (Khattat and Stewart 1975). In our study, abortion of reproductive organs occurred only at the R2–R3 growth stages, and in soybeans, Lima beans and green beans there is a similar restriction to early stages of raceme development (Baker *et al.* 1946; Broersma and Luckmann 1970; Khattat and Stewart 1975).

Abscission resulting in abortion may be a response to reduced flow of auxins from the pod (Osborne 1989; Ofir *et al.* 1993), or be a response to reduced photosynthate sink activity of the pod (Binnie and Clifford 1999). We observed that in cages with insects, aborted pods had internal lesions. Such lesions could, as hypothesized by Strong (1970) and Tingey and Pillemer (1977), lower the levels of auxins released by the pods, but could also reduce the pod's sink activity. In either mechanism, *Lygus* feeding is interfering with the normal regulation of abscission in the raceme, a process that occurs only during a critical period within 5 days of anthesis (Sage and Webster 1987). Our finding that pod abortion did not occur in later growth stages supports the hypothesis that the elevated levels of abortion were not a direct response to *Lygus* feeding, but rather a consequence of that feeding modifying the plant's normal system of regulation of abscission during the critical period.

Necrosis of the vascular tissues in the placental and funiculus regions in pods at the R4–R5 stage occurred only in cages with *Lygus*. Developing seeds within pods receive minerals and photosynthates through these vascular tissues, and shortage of these resources results in abortion and collapse of fertilized ovules (Adams 1967). Hence, injury to the vascular tissues supplying nutrients is the most likely cause of shrivelling of seeds, which was very frequent at pod maturity when *Lygus* feeding had occurred at the R4–R5 stages. Seed shrivelling in response to *Lygus* feeding also occurs in Lima beans, *P. lunatus* (Baker *et al.* 1946). In *P. vulgaris*, shrivelling of seed is a normal phenomenon in

healthy pods, where the frequency of undeveloped seeds may exceed 90% for the basal seed position in the pod and be 10–30% in more distal positions (Harris 1915; Nakamura 1988).

Direct injury to seeds was prevalent in the R6–R7 stages and infrequent at the R4–R5 stages. The central pit of the lesions is likely to be the result of physical and enzymatic destruction of the cells of the testa and underlying cotyledon; the surrounding pigmentation probably arises from the oxidation of phenols through the plant's wound response (Hori 2000) or by salivary enzymes (Cooper *et al*. 2013). Such blemishes on seeds could make beans unsuitable for canning (United States Department of Agriculture 2001); ≥1% of blemished seed in a sample for grading results in grade reduction in Canada (Canadian Grain Commission 2019). Similar seed pitting occurs in Lima bean when *Lygus* feed on developing seeds (Baker *et al*. 1946; Elmore 1955).

Field diagnosis of *Lygus* injury and implications for economic loss

This study has identified the symptoms of *Lygus* injury to navy bean racemes that are observable within a few days of that injury occurring. Knowledge of these symptoms can allow for more accurate diagnosis of *Lygus* injury during field scouting in field beans. However, the presence of *Lygus* or of the injury they have caused does not necessarily predict economic loss.

Early in the growing season, *Lygus* adults are detectable in low numbers in field beans at growth stages up to R3 (Nagalingam and Holliday 2015). Although *Lygus* feeding induces abscission of reproductive structures at these growth stages, detached buds, flowers or small pods are not diagnostic of *Lygus* injury because of the prevalence of abscission of reproductive structures in healthy plants. Reliable signs of *Lygus* injury up to the R3 growth stage include brown pigmented feeding spots and associated tissue swelling or split lesions. However, it is not clear whether *Lygus* injury at these early growth stages causes economic loss. Plants of *P. vulgaris* respond to removal of early flowers by setting pods from later flowers (Binnie and Clifford 1981) and respond to removal of early pods by reduced frequency of abortion of later-developing pods (Tamas *et al*. 1979). Thus, the plant may compensate partially or completely for loss of some reproductive structures due to *Lygus* injury.

Following the arrival of *Lygus* adults, a generation of nymphs develops in field beans but, probably because of low sampling efficiency, only low numbers are detected in sweep net samples during the R4–R5 growth stages (Nagalingam and Holliday 2015). At these growth stages, funiculus and placental injury are characteristic, and external examination of developing pods for discolouration near the ventral suture, followed by internal examination for brown lesions near the vascular tissues, provides a reliable indicator of *Lygus* injury. The resulting shrivelling of seed is not definitive because many seeds fail to fill in the absence of *Lygus* (Harris 1915). Plants may compensate for *Lygus*-induced seed

shrivelling. Seed filling occurs within an environment regulated by nutrient competition (Adams 1967) and a relatively high proportion of seeds fail to fill (Harris 1915; Nakamura 1988); *Lygus*-induced removal of some seeds from the competition could allow filling of unaffected seeds that would otherwise fail to fill. It is difficult to assess whether *Lygus*-induced seed shrivelling increases the proportion of shrivelled seed at harvest, as shrivelled seed is likely to be under-represented in harvested samples following threshing and cleaning.

Late in the growing season, there are peaks of sweep net catches of nymphs and adults that occur at R6–R7 (Nagalingam and Holliday 2015). At these growth stages, pods with exterior penetration points and containing seeds with direct injury to the testa are diagnostic for *Lygus* feeding. Reduced grade of seeds because of seed pitting is likely to lead to economic loss for bean producers. Retrospective assessments of *Lygus* injury based on harvested seed can clearly show evidence of direct *Lygus* injury to seeds, which produces seed pits.

Acknowledgements

We thank D. Holder and L. Babey for technical help, A. Leroux, T. Dickson, and L. Peixoto for assistance with *Lygus* colony establishment, and Dr. A. Hou and Dr. P. Balasubramanian of Agriculture and Agri-Food Canada for technical advice. Funding was provided by Manitoba Pulse and Soybean Growers, Manitoba's Agri-Food Research and Development Initiative, and the University of Manitoba.

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