Observations of selective feeding of the aphid, *Sitobion yakini* (Eastop) on leaf blades of barley (Hordeum vulgare L.)

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ABSTRACT

Penetration of leaves of barley (*Hordeum vulgare* L.) plants grown under normal conditions and those exposed to physiological stress, by the aphid, *Sitobion yakini* was investigated using light and electron microscope techniques. This was carried out to determine if there was evidence of preferential feeding on either thin- or thick-walled sieve tubes in the barley leaf.

Under both stress and non-stress conditions, preliminary results of an electron microscope investigation showed that penetration of the epidermis and mesophyll was largely intercellular, becoming partly intercellular and in part intracellular inside the vascular bundle. A total of 317 stylets and stylet tracks were encountered during the examination of 2000 serial sections. In non-stressed plant material, 293 (92%) terminated in thin-walled sieve tubes and only 24 (8%) in thick-walled sieve tubes. Investigation of 1000 serial sections using stressed plant material showed, that 84 of 89 (94%) stylets and stylet tracks encountered terminated in thin-walled sieve tubes. Furthermore, 90 of 94 (96%) stylets and stylet tracks encountered in 1000 serial sections from the second experiment of control non-stressed plant material (control) terminated in thin-walled sieve tubes. The thin-walled sieve tubes were significantly more visited (probed) by the aphid than the thick-walled sieve tubes. Under stress conditions, 50 of 89 (56%) stylets and stylet tracks which terminated in thin-walled sieve tubes were associated with the small longitudinal bundles. Under normal conditions, 65 of 94 (69%) stylets and stylet tracks which terminated in thin-walled sieve tubes were associated with the small longitudinal bundles. There were no significant differences on the number of probes of sieve elements between the two treatments. These data suggest that the aphid *S. yakini* feeds preferentially on the thin-walled sieve tubes of the small longitudinal vascular bundles in plants grown

under normal conditions and those exposed to physiological stress, such as water stress. This further suggests that the thin-walled sieve tubes in barley leaf blades are more attractive to the aphid and are probably most functional in terms of phloem loading and transport.

CHAPTER 1: INTRODUCTION

A wealth of information dealing with phloem structure and function is available at the light and electron microscope level. During the past two decades, considerable effort has been devoted to advancing our knowledge on the structure and function relationship of monocotyledonous phloem. A number of studies have shown that barley and other members of the Poaceae, including wheat, sugarcane, maize and southern African grasses such as *Themeda triandra,* contain both thin- and thick-walled sieve tubes, at least in the intermediate and small leaf-blade bundles (see Dannenhoffer, Ebert and Evert 1990; Kuo and O'Brien 1974; Colbert and Evert 1982; Walsh 1974; Botha, Evert, Cross and Marshall 1982b and references cited therein). Within monocots studied to date, these are classified as first-formed and late-formed metaphloem.

The first-formed metaphloem consists of sieve tubes and associated companion cells. These are called thin-walled sieve tubes, because their walls are thin compared to the walls of the other late-formed sieve tubes which form towards the end of the differentiation cycle. These last-formed metaphloem elements differentiate next to the metaxylem vessels. According to available literature, these late-formed sieve tubes lack companion cells (see Evert, Eschrich and Heyser 1978, Evert, Russin and Botha 1996, Botha and Cross 1997 and literature cited). The thick-walled sieve tubes in the wheat leaf are lignified (Kuo and O'Brien 1974) but those in the leaves of sugarcane, maize and *Themeda triandra* apparently are not.

Studies undertaken of the small and intermediate bundles of the barley leaf by Evert, Russin and Botha (1996) and Botha and Cross (1997) showed that the thick-walled sieve tubes have very few plasmodesmata in their common walls with other cell types. These authors found that plasmodesmatal connections between sieve tube-companion cell complexes and other cell types of the leaf, including vascular parenchyma cells and thick-walled sieve tubes, were also rare. The authors therefore concluded that both the sieve tube-companion cell complexes and the thick-walled sieve tubes may based on plasmodesmatal frequencies be virtually symplastically isolated from the rest of the leaf. This contrasts sharply with that in maize and sugarcane bundles, where only the sieve tube-companion cell complexes are symplastically isolated (Evert, Eschrich and Heyser 1978, Robinson-Beers & Evert, 1991).

Several authors have suggested that the reliance upon plasmodesmatal frequency alone as a determinant of cell–to-cell transport (see Botha and van Bel 1992, Evert *et al*. 1996 and Botha and Cross 1997) should be treated with caution when interpreting results. Botha and van Bel (1992) argued that plasmodesmatal frequency data merely gives an indication of the maximum potential pathway of symplastic transport and does not take the transport capacity of the plasmodesmata into account. The use of fluorescent dyes such as Lucifer yellow, which are able to move symplastically, has provided more information on symplastic continuity. Where plasmodesmatal frequency studies are coupled with microinjection and microiontophoretic studies, they provide more meaningful information about cell-to-cell transport in the phloem.

Lucifer yellow (LYCH) was used by Farrar, van der Schoot, Drent and van Bel (1992) to demonstrate symplastic continuity in mature leaves of barley. These investigators concluded that LYCH injected into parenchymatous bundle sheath and mestome sheath cells, was transferred symplastically to the sieve tubes of intermediate bundles. In a subsequent study, Botha and Cross (1997) specifically focused on plasmodesmatal frequency in relation to short distance transport and phloem loading in barley. Plasmodesmatal frequency data, together with electrophysiological and intercellular microiontophoretic injection of LYCH suggested a two-domain phloem loading strategy in barley, confirming the near or complete isolation of the sieve tube-companion cell complex from the vascular parenchyma.

Based on the above studies it is clear that there is support for the concept of a disjunction between the sieve tube-companion cell complex and the thick-walled sieve tube and their associated vascular parenchyma cells. However, even given these studies there is still little real evidence for functionally different roles for the thin- and thick-walled sieve tubes in barley or any other grass for that matter.

1.1 Speculation about the function of the thin- and thick-walled sieve tubes

The presence of these two types of sieve tubes in grass leaves has led to speculation about their possible role in phloem loading and transport. Kuo and O'Brien (1974) first suggested that the thick-walled sieve tubes in *Triticum aestivum* (wheat) may be specialised for either long distance transport, or serve as temporary storage reservoirs for sugar in excess of what can be transported by the thin-walled types of sieve tubes. However, results from a microautoradiographic study of the transport of ^{14}C-photosynthates transport do not support a functional role for the thick-walled sieve tubes, which in this species is involved neither in storage nor directly in the transport of photosynthates (Cartwright, Lush and Canny, 1977).

Subsequently Evert *et al.* (1978) suggested that the thick-walled sieve tubes in *Zea mays* may play a role in the retrieval of solutes entering the leaf apoplast from the transpiration stream. This suggestion was based on the observed close spatial association between the thick-walled sieve tubes and the metaxylem vessels, as well as on their possession of plasmalemma tubules, which apparently greatly increase the apoplast-symplast interface (Evert, Eschrich, Neuberger and Eichhorn 1977). Evert and Mierzwa (1989) further suggested that in barley, the presence of a pectin-rich wall layer permeated with microvilli-like evaginations of the plasmalemma should greatly facilitate the movement and uptake of water and solutes across the cell wall-plasmalemma interface.

Microautoradiographic studies of phloem loading and transport in the leaf of maize (Fritz, Evert and Heyser 1983) showed that the thin-walled sieve tubes accumulated sucrose and photosynthate from the apoplast, without the companion cells serving as intermediary cells and that the thin-walled sieve tubes are primarily involved with the uptake of photosynthate. From this study, it is apparent that the thick-walled sieve tubes could receive photosynthate that was transferred to the thick-walled sieve tubes, after accumulation by contiguous vascular parenchyma cells. The authors also suggested that they could accumulate assimilates through retrieval from the xylem, into which solutes could have leaked from the phloem free space. However, a cautionary note needs to be sounded here as the experiments were conducted on detached leaves. The microautoradiographic study by Fritz and Evert (1983) further suggested assimilates could be transferred from the thick-walled sieve tubes to the thin-walled sieve tubes and were thus unlikely to involved in long distance transport

Free-space marker studies on the leaves of *T. triandra, Z. mays, Saccharum officinarum* and *Bromus uniloides* (Botha, Evert, Cross and Marshall 1982a, Evert, Botha and Mierzwa 1985, Botha and Evert 1986) showed the accumulation of Prussian blue crystals in the cell walls of both thin- and thick-walled sieve tubes, demonstrating the ease with which water moves from the lumina of the vessels to the walls in both thin- and thick-walled sieve tubes and their associated apoplast. The implication of the ease of uptake or transport of Prussian blue suggests that the cell walls of the thick-walled sieve tubes and the thin-walled sieve tubes are freely permeable to water and solute, and thus do not or should not impede solute transfer across these cell wall -interface.

Even though there is evidence that both thin- and thick-walled sieve tubes have been shown to be capable of assimilate uptake and transfer (Fritz, Evert and Heyser 1983) and again that the cell walls of thin- and thick-walled sieve tubes are apparently freely permeable to solutes (Botha and Evert 1986 and literature cited) there is no real evidence of a long-term function for the thick-walled sieve tubes in grasses. Clearly, alternative methods need to be explored to determine the role of the thick-walled sieve tubes in plants such as barley.

1.2 The role of aphids in phloem research

1.2.1 Aphid feeding and nutrition

Aphids are small insects which feed by inserting their stylets into the plant tissue, where they feed on cell sap. Several studies of stylet anatomy carried out at electron microscope level (see Evert, Eschrich, Medler and Alfieri 1968, Mittler 1957, Botha, Bornman, Carter and Heeg 1972) demonstrated their remarkable adaptation for puncturing and penetrating plant tissues. The mandibular stylets have been found to possess hook-like projections on their surfaces and the maxillary stylets possess a series of lateral tooth-like projections. These features are presumed to play a part in the penetration of the stylet group into the plant tissue.

As an aphid inserts its stylets into plant tissue, it secretes a stylet sheath around the stylets. Having punctured a functional sieve element (which is assumed to be under full turgor) the aphid is virtually force-fed and is believed to remove chiefly amino acids and sugars for its own dietary needs, excreting the non-essential liquid as well as any undigested sap as honeydew. When the aphid withdraws its stylets the stylet sheath remains in the plant tissues, and indicates the exact passage taken by the stylets during feeding. The point where the sheath terminates indicates the tissue on which the aphid has fed and this termination usually proves to be sieve tubes (see Evert, Eschrich, Medler and Alfeiri 1968; Botha, Evert and Walmsley 1975a,b; Botha and Mabindisa 1977; Botha and Evert 1978; Bornman and Botha 1973; Prado and Tjallingii 1994; Caillaud and Niemeyer 1996 and literature cited).

Aphids mostly aggregate on parts of a plant where food is of high quality (Kennedy and Booth 1951). In other words, they select their feeding sites according to the quantity and quality of the food that should be yielded at that site. It has been shown, using membrane-feeding systems that the probing behaviour of some aphid species and feeding can be influenced by plant-specific chemicals which are found in parenchyma cells (for further details see Montgomery and Arn 1974). High levels of sucrose, amino acids and inorganic ions, (which are characteristic constituents of phloem sap) may provide the stimuli by which phloem feeding aphids “recognise” sieve elements (Mittler and Dadd 1965). The suggestion is that phloem feeders therefore feed at the site of high sucrose loading, that is, on the functional phloem. Changes in the distribution of an aphid with time could therefore indicate changes in concentration gradients within the phloem sap itself.

Clearly aphid feeding involves not only the phloem sap, but also other plant tissues overlying and associated with the vascular tissues, which may interact with the insect mouth parts and therefore play an important role in the final acceptance of plants as suitable hosts.

Recently developed methods for studying the feeding behaviour of aphids include electronic feeding monitors which give information about the activities of aphids while their stylets are inside plant tissue. Studies carried out using the above method demonstrate that aphids are capable of two activities during sieve element puncture (Prado and Tjallingii 1994): 1) salivary excretion into the sieve element where the fluid filled canal does not allow saliva to be ingested, 2) passive ingestion of sieve element sap which is mixed with the concurrently secreted saliva. The secreted saliva in the latter case does not reach the plant (sieve element) but is mixed with the sap. The first example is suggested in this thesis to refer to a non-functional feeding environment and the second example referring to a functional feeding environment.

Many aphids show a non-random distribution over the surfaces of leaves on which they feed. Their location is related to differences in the size of the leaf veins from which the aphids feed (Kidd 1976). Larger aphids tend to settle on larger veins, while the smaller aphids settle on the smaller veins. In some species (see Gibson 1972, Elliot and Hodgson 1996) this pattern of distribution is simply the result of the stylets of small aphids being too short to reach the deeper phloem of the larger veins, while the larger aphids with their longer stylets are able to accomplish this.

1.2.2 Investigation of phloem sieve tube physiology, using feeding aphids and aphid stylectomy techniques.

From the earliest days of the study of phloem transport, investigators have attempted to probe the mysteries of the functional sieve-tube system by obtaining samples of the nutrient stream which moves through it. The dependence of aphids on phloem sap as a source of food was exploited over the years in a number of laboratories, in the attempt to solve the as yet, unknown issues related to phloem transport and sieve content. A popular technique which has been used over many years, is to sever the stylets of feeding aphids and collect the sap that exudes from the cut ends of the stylet bundle left embedded in the host plant. Kennedy and Mittler (1953) used this technique to determine the composition of the food digested by aphids. Weatherley, Peel and Hill (1958) used the technique to study sieve tube physiology. Recently, Pritchard (1996) has used this method to measure the osmotic pressure of functional sieve tubes. Clearly, this technique has been used extensively in an attempt to rationalise some of the many problems and major controversies associated with phloem translocation.

Whole aphids have frequently been used to collect sieve tube sap in the form of excreted honeydew (Ho and Peel 1969). Studies undertaken by several authors (Esau, Namba and Rasa 1961, Evert *et al.* 1968, Bornman and Botha 1973, Botha *et al*. 1975a, b, Botha and Evert, 1978,) demonstrated that aphids are useful tools in the study of functional phloem tissue. The use of live, feeding aphids was necessary in these experiments as this allowed the aphids to settle and feed undisturbed. As a result thus more information on stylet position as well as about the effects of prolonged feeding by the aphids could be obtained.

It is clear that feeding aphid colonies present opportunities to examine potential functionality of specific phloem groups as was the case in studies by Botha, Evert and Walmsley (1975a,b); Botha and Mabindisa (1977); Botha, Malcom and Evert (1977); Botha and Evert (1978), where preferential feeding habits were revealed.

Given that grasses have thick- and thin-walled sieve tubes and given the controversy surrounding their potential roles in retrieval, loading and transport it became clear that some of the as yet, unanswered questions relating to function could possibly be answered, by examining preferential feeding sites of aphids in leaf blade bundles in a grass such as barley.

1.3 Research Objectives

The study of feeding habit of aphids on barley, conducted by Evert, Eschrich, Eichhorn and Limbach (1973) carried out at the electron microscope level of stylet penetration by the maize leaf aphid, *Rhopalosiphum maidis* showed that the aphid preferentially probed from the adaxial surface of the leaf. Whilst a great deal of useful information was gained from this study, the authors did not indicate which (thin- or thick-walled) sieve tubes were penetrated.

Aphid feeding has been equated to ‘preference feeding’ and this implies that it is the functional phloem which is penetrated. In addition the fact that there is no clearly-defined or recognised role for the thick-walled sieve tubes in grasses such as barley, it was decided that this could be investigated appropriately using feeding aphid colonies.

Therefore, the hypothesis upon which the research was based was that there may be no functional role for the thick-walled sieve tube in barley grown under normal (unstressed) conditions or in plants which had been exposed to physiological stress (droughting). In order to test this colonies of aphids were allowed to feed and subsequently sacrificed in order to determine if there was any preferential feeding pattern and thereby to determine the role of the thick- and thin-walled sieve tubes in leaves of barley.

This study therefore focuses on the feeding pattern and point of termination of stylets and stylet tracks, in order to assess feeding preference of the aphid *Sitobion yakini.*

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant host material

Seeds of barley (*Hordeum vulgare* L. *cv*. Clipper) were pre-germinated in petri dishes and thereafter sown in acid washed river sand in plastic pots (diameter = 17cm). When the plants were two weeks old, colonies of the aphid, *Sitobion yakini* (Eastop) were transferred to them and the aphid-containing plants were kept in insect cages in the greenhouse. The aphid colonies were supplied with young barley plants every two weeks to ensure succulent hosts. Plants with suitably-established aphid colonies were selected for light and electron microscope investigation of the stylet penetration.

2.2 PEG induced water stress

Barley plants were grown in sand in plant pots in the greenhouse. They were irrigated with dilute Long-Ashton nutrient solution (see appendix A and Hewitt 1996) daily and allowed to drain freely. After 12 days (12 days after germination) the plants were divided into two groups: a control group and stressed group. The plants in the control group were watered and supplied with nutrients on a daily basis, whilst those in the stress group were subjected to a controlled low water potential by adding Polyethylene glycol (PEG 4000, Merk Pty Ltd, Midrand, South Africa) to the culture solution to give a final water potential of –0.8 MPa. The plant roots were allowed to adapt to the increase in osmotic stress by successive daily increment of –0.2 MPa to -0.8 MPa over 4 days. Osmotic stress was then maintained at –0.8 MPa for a further 10 days.

Table 2.1 Ratio of water (containing Long- Ashton nutrient solution) to PEG to yield ranges of osmotic stress.

Water (H_2O) (ml)	PEG (g)	MPa
100	9	-2
100	13	-4
100	17	-6
100	21	-8

2.3 Leaf water potential

The leaf water potential of fully expanded leaves was measured using a pressure chamber. Leaves were excised from the plant with a sharp razor blade and inserted into the pressure chamber with the cut surface protruding slightly through the rubber-sealing gasket. Pressure was then increased gradually in the chamber by adding air from a compressed gas source. The pressure was increased until water in the xylem first appeared at the cut surface of the leaf. The accurate determination of this end point was aided by the use of a high-magnification hand lens. The balance pressure in the chamber at this end point, taken as a negative value, equals the apoplastic value in the leaf, which in turn equals the symplastic value of the water potential.

2.4 Light microscopy

Aphids were killed *in situ* by rapidly exposing them to an atmosphere of acrolein (Merk Chemicals, Germany). Leaf segments containing attached aphids were fixed in FAA for 24 hours. The leaf segments were then gently cut into smaller, more manageable pieces and dehydrated through an alcohol and tertiary butyl alcohol series. The material was then infiltrated with a number of changes of paraplast wax over three days, in an embedding oven at 60^0C. Blocks were mounted and trimmed and serial sections were cut at 15µm using a Minot rotary microtome (Leitz Wetzlar, Germany). Sections were stained in Safranin and Fast green. The stained sections were mounted onto slides using Canada Balsam and dried in an oven at 37^0C for three weeks. Serial sections were carefully examined for evidence of aphid feeding, by careful examination for stylet tracks or sheaths which are easily seen after staining the sections.

2.5 Electron microscopy

2.5.1 Transmission electron microscopy (TEM)

Some leaf blade segments with attached aphids were carefully cut into smaller pieces and fixed in 2.5% glutaraldehyde in 0.05M sodium cacoldylate buffer for 24 hours. The segments were washed in two 30 min. changes of 0.1M cacoldylate buffer and post-fixed in 1% osmium tetroxide in 0.1M cacoldylate buffer for 90 min. After post-fixation, pieces were rinsed in cold cacodylate buffer and dehydrated in a cold, graded ethanol series, followed by two 30 min. changes of propylene oxide. Embedment was in Araldite Taab 812 resin. Monitor sections of the leaf were cut with glass knives and mounted in water, on microslides and dried down at 60°C. Sections were stained in toluidine blue for 1 minute, rinsed in distilled water and then mounted under coverslips. Ultrathin sections (silver to gold) were cut using a diamond knife (Drukker, Netherlands) and were collected on 300 mesh copper grids (SPI suppliers, Philadelphia, USA). They were then stained in uranyl acetate and lead citrate. Sections were viewed using a JEOL JEM 1210 transmission electron microscope (JEOL, Tokyo, Japan).

2.6 Data analysis

All data collected were analysed using Statistica 99 software (Sta-soft, Incl). The significant differences between the number of times the aphid probes a sieve tube and/or vascular bundle is visited by were calculated using a One-way Analysis of Variance (ANOVA) and Chi^2- tests. The letters **a** and **b**, given in the statistical tables define the significance of the data for sieve elements and/ or bundles.

CHAPTER 3: RESULTS-AN EXAMINATION OF STYLET FEEDING ON BARLEY LEAF BLADES BY *S. YAKINI.*

3.1 Anatomy of leaf blade

The leaf blade of barley has typical Poaceae grass anatomy, with a system of longitudinal vascular strands interconnected by numerous transverse veins and vascular bundles widely separated by a loosely-arranged mesophyll (Dannenhoffer *et al.* 1990). The vascular bundles are surrounded by two sheaths, an inner mestome sheath, and an outer parenchymatous bundle sheath (Evert *et al*. 1996). Three orders of longitudinal vascular bundles (see Fig. 3.1) are present namely, large (first order), intermediate (second order) and small (third order). As in other grasses, large and intermediate bundles are associated with girders or strands of hypodermal sclerenchyma (Botha *et al*. 1982b, Dannenhoffer *et al.* 1990).

3.2 Cellular composition of vascular bundles

3.2.1 Large bundles

When viewed in transection, mature large bundles are characterised by the presence of a protoxylem lacuna and two large metaxylem vessels, one on either side of the lacuna (Fig. 3.2). The protophloem sieve tubes border the mestome sheath abaxially and are usually collapsed or obliterated in such bundles, leaving only functional metaphloem, which contains both thin- and thick-walled sieve tubes. Only the thin-walled sieve tubes have companion cells. In addition, the large bundles contain vascular parenchyma cells, some of which occur on the xylem side of the bundles, spatially associated with the vessels. On the phloem side of the bundle, some parenchyma cells abut both vessels and sieve tubes, whilst others abut only sieve tubes and their associated companion cells. These vascular parenchyma cells occupy most of the interface between the sieve tube-companion cell complexes and the mestome sheath. In addition, a layer of vascular parenchyma cells (usually only one cell wide) commonly occurs between the sieve tube-companion cell complexes and the thick-walled sieve tubes.

3.2.2 Intermediate bundles

The intermediate bundles are associated with girders or strands of hypodermal sclerenchyma which are often reduced and the strands are not always in contact with the vascular bundles. They lack protoxylem lacuna as well as the large metaxylem vessels which are characteristic of the large bundles only. Protophloem may be present, but in mature bundles these are mostly collapsed or obliterated. As described by Dannenhoffer *et al.* (1990), the metaphloem consists of early (thin-walled) metaphloem sieve tube-companion cell complexes and late (thick-walled) metaphloem sieve tubes tubes (open and filled circles, respectively, Fig. 3.3). Vascular parenchyma occupies most of the interface between the sieve tube-companion cell complexes and the mestome sheath and commonly separates the sieve tube-companion cell complexes from thick-walled sieve tubes.

3.2.3 Small bundles

Small bundles are generally smaller than the intermediate bundles (Fig. 3.4). They are similar in structure to the intermediate bundles and have often been classified as such merely by the absence of spatial association with hypodermal sclerenchyma (Botha *et al*. 1982b, Dannenhoffer *et al.* 1990). They contain vascular tissue composed of metaxylem and metaphloem. The metaphloem contains both thin- and thick-walled sieve tubes. The smallest bundles may, on occasion, lack thick-walled sieve tubes (Evert *et al.* 1996). The number of vascular parenchyma cells is reduced compared to the intermediate bundles.

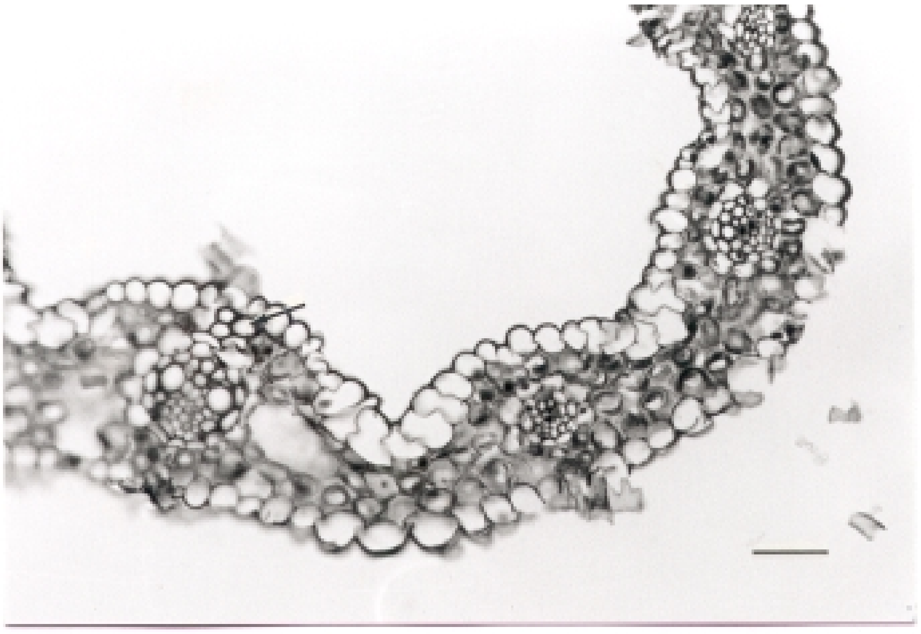


Fig. 3.1 Transverse section of barley leaf. This portion of the leaf contains a large, intermediate and small vascular bundle. Unlabelled arrows point to hypodermal sclerenchyma. Bar = 10µm

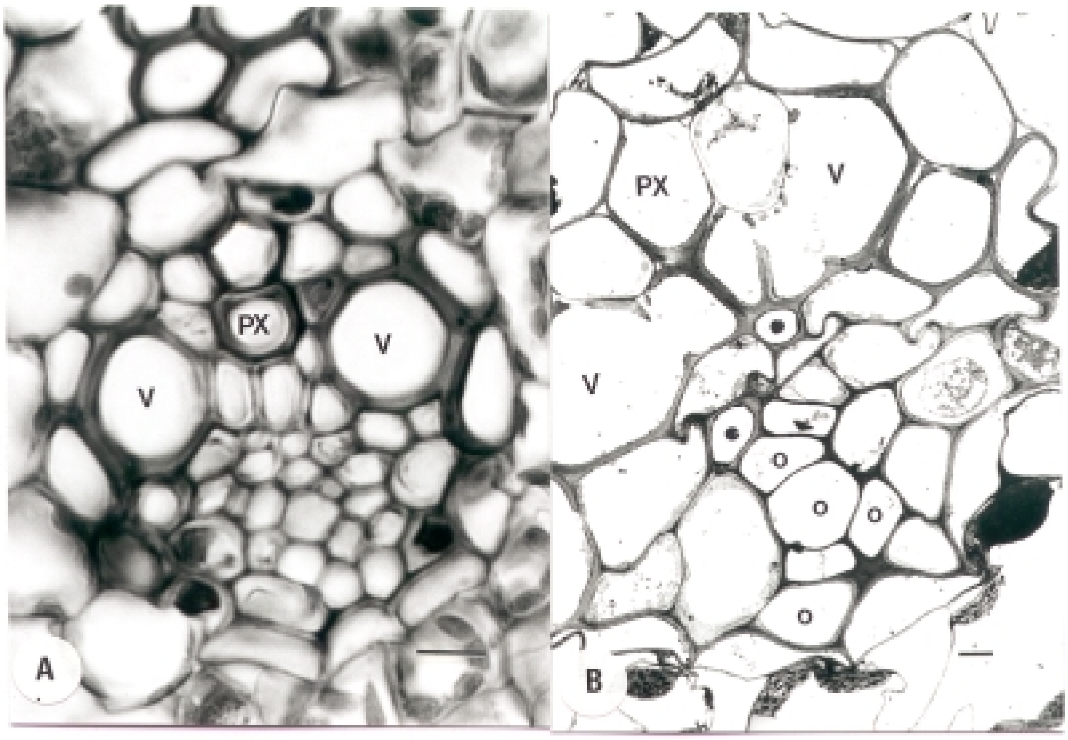


Fig. 3.2A light, **Fig. 3.2B** transmission electron micrograph (TEM) showing a large vascular bundle in transection. Such bundles are characterised by the presence of large metaxylem vessels (V), one on either side of the protoxylem (PX) (Fig. 3.2 A). Fig 3.2 (B) shows an electron micrograph of portion of large bundle. ×1500. Thick-walled sieve tubes (solid dot) occur next to the xylem and thin-walled sieve tubes (open circles) below. Bar = 10μm (A), bar = 2μm (B)

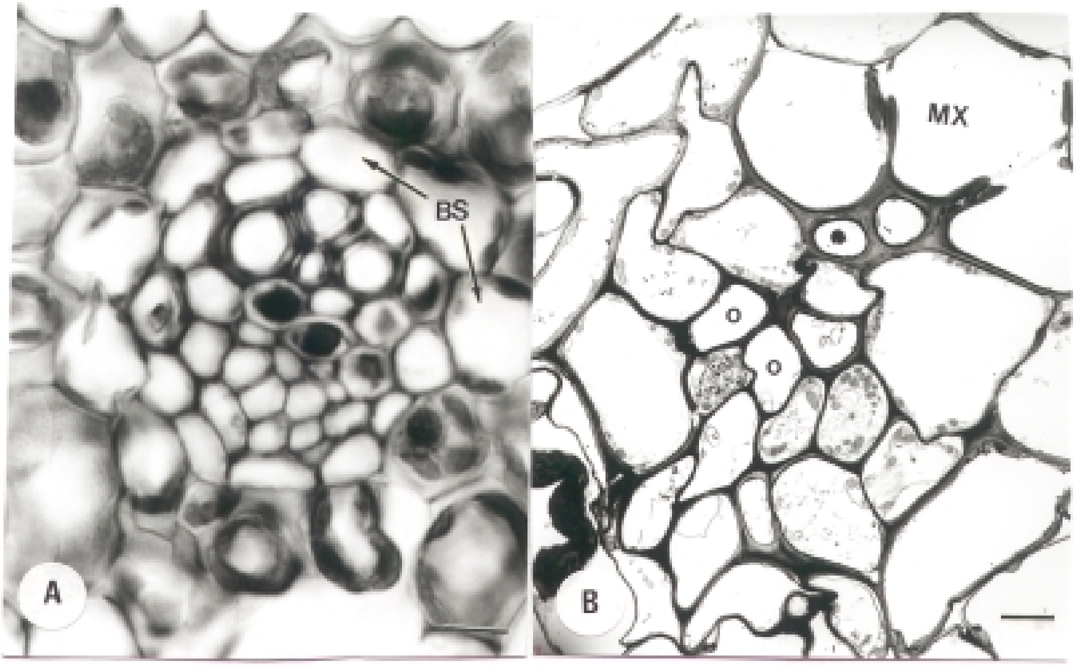


Fig. 3.3A light, Fig. **3.3B** transmission electron micrograph (TEM-×2000) showing an intermediate vascular bundle in transection. Note that these bundles lack large metaxylem vessels (MX) and protoxylem. Both thick (solid dot) and thin-walled sieve tubes (open circles) are present. BS= bundle sheath. Bar = 10µm (A), bar = 2µm (B)

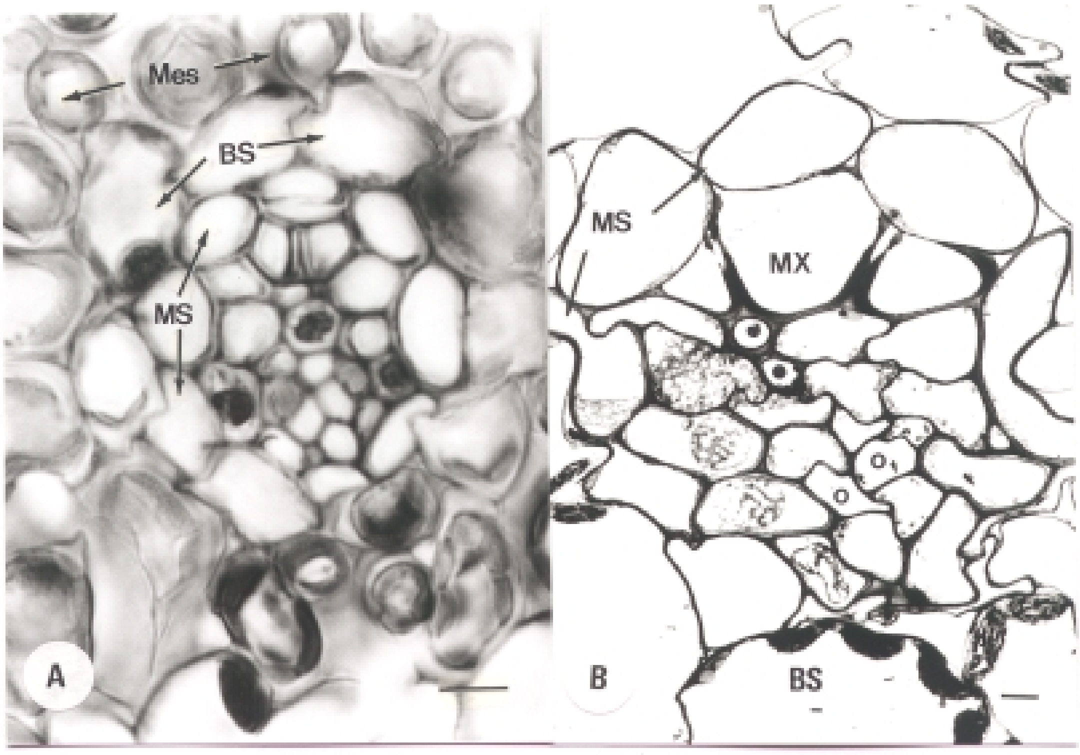


Fig. 3.4A light, **Fig. 3.4B** transmission electron micrograph (TEM-×1500) showing a small vascular bundle in transection. Two thick-walled sieve tubes (solid dots) occur adjacent to the metaxylem (MX). A parenchymatous bundle sheath surrounds a mesostome sheath (MS) and undifferentiated mesophyll (Mes) surrounds this vascular bundle. Bar = 10µm (A), bar = 2µm (B)

3.3 Aphid feeding on unstressed plant material: **Penetration of the leaf blade by *S. yakini* :**

Information on the manner of penetration of the leaf blade by the aphid was obtained by following the pathway of the stylet tracks or sheaths in serial transverse sections of the host tissue microscopically. Stylet tracks were examined for point of origin (i.e., from the ad- or abaxial leaf surface) to the point of termination (i.e., in the thin- or thick-walled sieve tubes). The principal data obtained during this study are presented in Tables 3.1 and 3.2 and Figs. 3.5-3.10.

By far the greater majority (92%) of the stylet tracks encountered during this study, terminated in the thin-walled sieve tubes (Table 3.1). In addition, 16 of 17 pairs of stylets encountered were lodged in the thin-walled sieve tubes and only one pair in the thick-walled sieve tubes (Table 3.2). Statistical analysis of results show a highly significant difference between thin- and thick-walled sieve tubes for each plant and for the total of all the plants (Tab. 3.3). The thin-walled sieve tubes (SE, 48.833 (± 2.00) are significantly more visited by the aphid than the thick-walled sieve tubes (TWSE, 10.147 (± 1.945).

Table 3.1: Distribution of stylets and stylet tracks/sheaths of *Sitobion yakini* feeding from longitudinal bundles of the leaf blades of barley.

Sieve element	Small VB	Int. VB	Large VB	# terminations on SE & TWSE	% Termination
SE	142	77	74	293	92
TWSE	0	6	8	24	8
Total	142	83	82	317	100

Note: Based on examination of 2000 serial sections.

Int. = intermediate, SE = Thin-walled sieve tubes, TWSE = Thick-walled sieve tubes, VB = vascular bundle.

Table 3.2. Origin and distribution of stylets and stylet tracks/sheaths of *Sitobion yakini* after penetration of the leaf blades of barley.

Observation	SOURCE	Termination	Total
STYLETS	Adaxial Epidermis	SE	9
		TWSE	1
Total			10
	Abaxial Epidermis	SE	7
		TWSE	0
Total			7
STYLET TRACKS/SHEATHS	Adaxial Epidermis	SE	93
		TWSE	9
Total			102
	Abaxial Epidermis	SE	184
		TWSE	14
Total			198

Note: Data based on examination of 2000 serial sections.

Table 3.3 The number of times the thin (SE) and thick (TWSE) walled sieve tubes are visited, with the mean and standard deviation. Chi^2-test level of significance; *** P<0.005. The letters a and b define the significant difference between the data for the sieve elements.

Replicate	SE	TWSE
1	65	9
2	57	8
3	46	5
4	38	1
5	45	0
6	42	1
Mean	48.833 (± 2.00) ^{a} ***	10.147 (± 1.945) ^{b}
Total	293***	24

Table 3.4. Results from one-way ANOVA for the variation between (A) and within (B) the number of times a sieve element (thin- (SE) or thick-walled (TWSE) and the bundles (small, intermediate, large) are visited. Level of significance One-Way-ANOVA: * *P*<0.05, ** *P*<0.01, * *P*<0.005. The letters a and b define the significant difference between the data for the sieve elements and/ or bundles.**

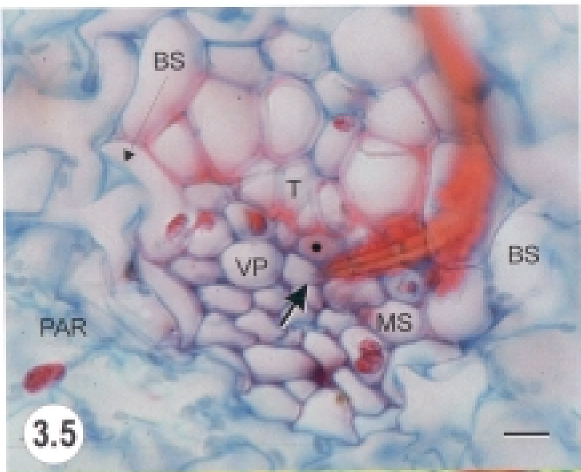
A	**Sieve elements**		Bundles			**P**
	SE	TWSE	Small	Inter.	Large	
Sieve elements	97. 67 (± 9.21) ^{a}	8.00 (± 4.58) ^{b}	-	-	-	***
Bundles	-	-	71.00 (± 50.21)	41.50 (± 25.10)	46.00 (± 19.80)	n.s.

B	Sieve elements		Bundles			**P**
	SE	TWSE	Small	Intermediate	Large	
Small	142 ^{a}	0 ^{b}	-	-	-	***
Intermediate	77 ^{a}	6 ^{b}	-	-	-	***
Large	74 ^{a}	18 ^{b}	-	-	-	***
SE	-	-	142 ^{a}	77 ^{b}	74 ^{b}	***
TWSE	-	-	0 ^{b}	6 ^{b}	18 ^{a}	***

Commonly, the stylets approached the veins obliquely, not directly from above or below. Branching of stylet tracks was relatively infrequent. Where branching occurred it was mostly either near to or in the bundles themselves (see Fig. 3.6, 3.8-3.10). Figure 3.10 shows the termination of a stylet within a thin-walled sieve tube. Examination of serial sections revealed that the aphid which formed this sheath first bypassed the bundle, entering the mesophyll on the upper side of the small vein, as shown by the salivary sheath left behind (unlabelled arrowheads point to unsuccessful probes). Partly withdrawing its stylet, the aphid then probed downwards by reflexing its stylets through approximately 90^0, sequentially it then penetrated the bundle sheath cell, a metaxylem vessel, then probed the thick-walled sieve tube and adjacent parenchymatous elements, before penetrating the thin-walled sieve tube below (arrow points to thin walled sieve tube containing a stylet tip). The thin-walled sieve tube contained unoccluded maxillary stylet tips (arrow points to tips of the stylets) which can be seen just protruding into the lumen of the thin-walled sieve tube. The lack of saliva deposition within the sieve tube, and the presence of open stylet tips inside the sieve tube is indicative of active feeding within this small vein (Evert *et al*. 1968, Botha *et al*. 1975a, Prado and Tjallingii 1994).

In all successful probes of the phloem tissue only the tips of the maxillary stylets were inserted into the sieve tubes being fed upon (see Fig. 3.5-3.7and 3.10). Fig. 3.7 shows the only successful probe of the thick-walled sieve tubes in which the stylets were still present. In this case, the aphid probed the metaxylem intercellularly, before penetrating the thick-walled sieve tube. This micrograph shows that only the tips of the maxillary stylets (arrowhead) entered the thick-walled sieve tube. They are separated and free of salivary material, suggesting active feeding prior to the aphid being killed.

Unlike the unoccluded stylets (Figs. 3.5-3.7 & 10), the stylets in Fig. 3.8 and 3.9 were surrounded by salivary material. It is likely that the aphid began to withdraw its stylets during manipulation of the leaf and, having been disturbed, may have ejected saliva into the sieve tube in question. It was therefore not possible to determine whether the aphid was feeding at the time it was killed. Penetration of several sieve tubes can be seen in the intermediate bundle (Fig. 3.9). Two, apparently unsuccessful, probes can be seen inside this intermediate bundle. The aphid first probed a bundle sheath cell intercellularly (arrowheads) and then continued through the central file of thin-walled sieve tubes, but does not appear to have probed the thick-walled sieve tube, but terminated at a thin-walled sieve tube. Fig. 3.8 shows a similar probe, where the aphid first penetrated the bundle sheath cells (apparently intercellularly) and then a metaxylem vessel and subsequently, a thick-walled sieve tube (solid dot) and ultimately, several sieve elements, before terminating in one of these (star points to terminal probe).



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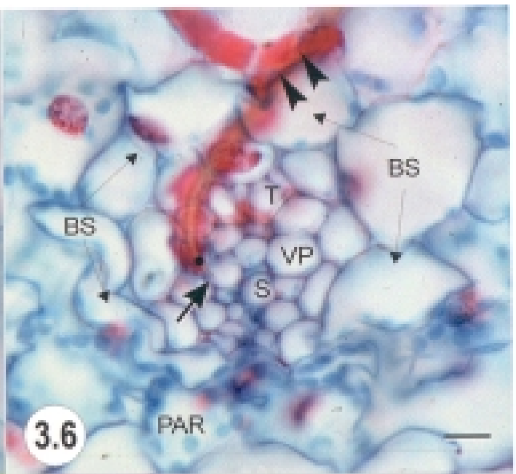

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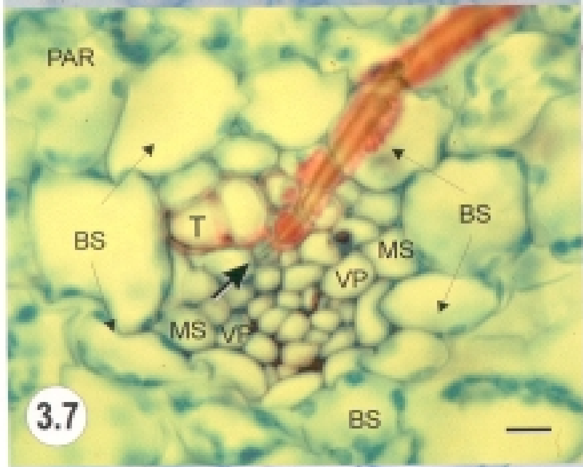

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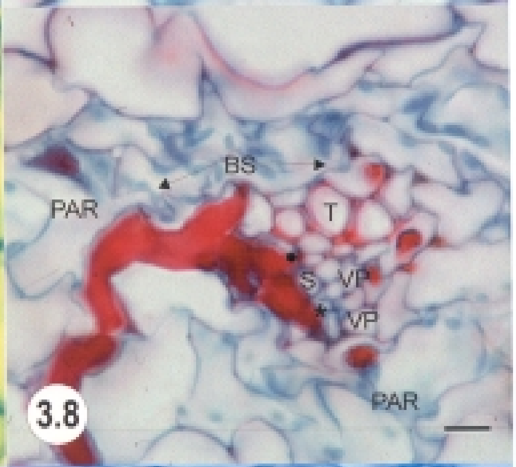

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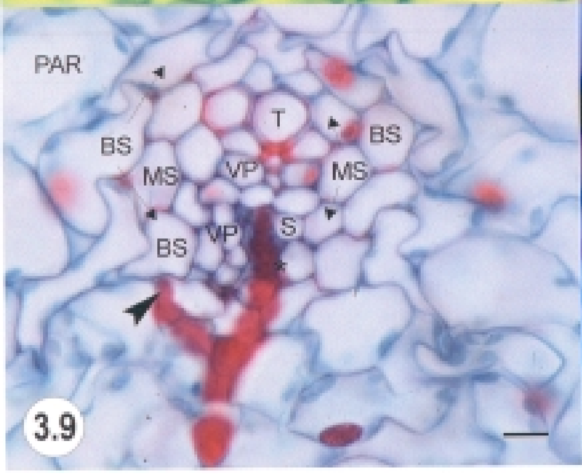

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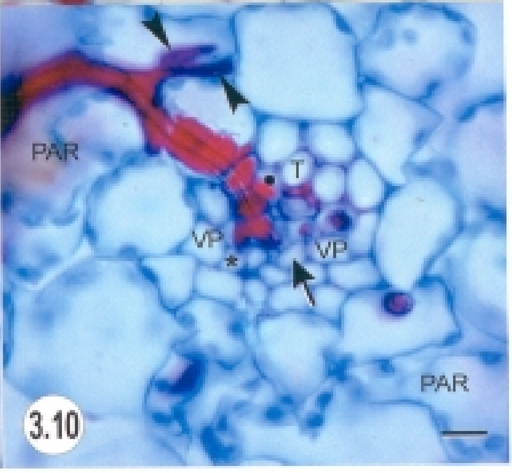

3.10

Figs.3.5-3.10. Show penetration of vascular tissue by *Sitobion yakini.* Scale bars = 10 µm.

Fig. 3.5. Shows penetration of a large vascular bundle, from the adaxial leaf surface. The aphid's stylet tips are visible (unlabelled arrow) just beneath a thick-walled sieve tube (solid dot). T = tracheary element; BS = bundle sheath; MS = mestome sheath; VP = vascular parenchyma; PAR = mesophyll parenchyma.

Fig. 3.6. Shows penetration by stylets of a thin-walled sieve tube, in a small vascular bundle. The tips are lodged within the sieve tube (unlabelled arrow). Lack of saliva associated with tips, suggests aphid was feeding from a functional sieve tube. S = sieve tube.

Fig. 3.7. Penetration of a thick-walled sieve tube. The aphid probed the xylem both intercellularly and intracellularly, before penetrating the thick-walled sieve tube. Stylet tips (unlabelled arrow) terminate within the thick-walled sieve tube. The tips are open and free of salivary material.

Figs 3.8-9. Show penetration of a small (Fig. 8) and intermediate (Fig. 9) leaf blade bundle. Both penetrations originated from the abaxial side of the leaf. **Fig. 3.8**. Aphid penetrated this vascular bundle from the side, re-curved its tips, and successively penetrated a thick-walled sieve tube (solid dot) and then sequentially probed all remaining thin-walled sieve tubes, terminating at a functional thin-walled sieve tube. Star points to a terminal probe. Lack of salivary material suggests that the aphid fed from this sieve tube. **Fig. 3.9.**shows a branched stylet track. After penetrating the bundle sheath to the left, the aphid pierced successive thin-walled sieve tubes. Termination at an unoccluded sieve element, suggests that the aphid fed from this functional element.

Fig. 3.10. Aphid stylets extending from the abaxial surface of the leaf, and terminating in a thin-walled sieve tube. The aphid first probed a mesophyll cell (unlabelled arrowheads) then continued downwards through bundle sheath and parenchyma cell, before terminating in the thin-walled sieve tube (unlabelled arrow points to stylets within sieve tube).

The majority of stylet probes terminating in the thin-walled sieve tubes were initiated from the abaxial surface of the leaf (see Table 2).

Of a total of 317 stylets and stylet tracks encountered during the course of this investigation, 142 were associated with the small vascular bundles, and 83 and 84 with large and intermediate bundles respectively. Results from One-way ANOVA for the variation between the two kinds of sieve tubes and the three kinds of vascular bundles (Table 3.4a) and within each vascular bundle (Table 3.4b) show the number of times a sieve tube (thin- or thick-walled) and the vascular bundles (small, intermediate and large) are visited by the aphid, does not show any significant difference between the vascular bundles. This surprising result may in part be due to the high standard deviation for the small bundle. It is important to note that the small bundles in the barley leaf-blade, contain only two-to three thin-walled sieve tubes, whilst the intermediate and large bundles contain more. The aphids thus aim at a smaller cross-sectional area of (presumably) functional phloem when they visit the small bundles, than would be the case with the intermediate and large vascular bundles.

3.4 Aphid feeding on stressed plant material

It is well known that plant water stress influences the phloem loading process in leaves of most higher plants (Vaadia, Raney and Hagan 1961 and literature cited therin). As a result it was decided to examine the potential involvement of the thick-walled sieve tubes in assimilate translocation under physiological drought stress. The level of stress was determined using water potential measurements to ensure that plants onto which the aphids were to be introduced were stressed.

Among the several effects on the physiology of plants through shortage of water two have marked effect, first, the loss of phloem pressure and secondly the enrichment of the phloem sap with soluble nitrogen through proteolysis, which may affect the food quality and supply of feeding aphids (Kennedy *et al*. 1958). As aphids depend largely on phloem pressure during feeding, they could be disadvantaged if the flow rate was diminished, as the volume ingested would be reduced concomitantly.

3.4.1 Penetration of leaves of stressed barley plant by stylets of S. yakini.

In a new series of experiments including control and stressed plants, penetration of barley leaf blades was examined identically to the data referred to in Table 3.1.

Of the 89 stylets and stylet sheaths encountered in leaf sections of water stressed plants 84 (94%) terminated in the thin-walled sieve tubes and the remaining 5 (6%) in the thick-walled sieve tubes (Table 3.5), compared to 90 of 94 (96%) in well-watered plants and the 4 (4%) which terminated in the thick-walled sieve tubes.

Surprisingly, the data presented in Table 3.5 and 3.6 show that there is no significant difference between the unstressed and stressed plant material with respect to feeding preference by *S. yakini*. Of interest is the fact that there is little difference between the data presented for the first feeding experiment (Table 3.1) and the subsequent one, also on unstressed plants (Table 3.5). For example, penetration of thin-walled sieve tubes was 92% and 96% respectively, and thick-walled sieve tubes was 8% and 4% respectively in samples of 2000 and 1000 serial sections, respectively. Table 3.6 shows an analysis for the variation between the two treatments in which there is clearly no significant difference in the number of times the thin- (21.0 and 22.5) or thick-walled sieve tubes were visited.

Of the 89 stylets and stylet tracks observed, 50 were associated with the small vascular bundles (Table 3.5) and the remaining 16 and 23 associated with the intermediate and large bundles, respectively. Also, in well-watered plants 65 of 94 stylets and stylet tracks observed were associated with the small vascular bundles (Table 3.5). In addition, the majority of stylets and stylet tracks which terminated in thin-walled sieve tubes of water stressed leaves were initiated from the adaxial surface of the leaf, compared to the majority observed in well watered plants which were initiated from the abaxial surface of the leaf. In water-stressed plants (Table 3.7) 61% of the probes terminating in thin-walled sieve tubes originated from the adaxial leaf surface compared to 22% in well-watered plants. In contrast, 31% of the probes terminating in thin-walled sieve tubes originated from the abaxial leaf surface compared to 78% in well-watered plants.

Table 3.5: Distribution of stylets and stylet tracks/sheaths of *Sitobion yakini* in the longitudinal bundles of the leaf blades of Barley.

Growth conditions	Type of se	Small VB	Int. VB	Large VB	Total # terminations, SE &TWSE	% termination
Water-stressed	SE	50	14	20	84	94
	TWSE	0	2	3	5	6
Totals		50	16	23	89	**100**
Well-watered	SE	65	10	15	90	96
	TWSE	0	1	3	4	4
Totals		65	11	18	94	100

Note: Based on examination of 2000 serial sections.

Int. = intermediate, SE = Thin-walled sieve tubes, TWSE = Thick-walled sieve tubes, VB = vascular bundles.

Table 3.6 Results of a one-way ANOVA for the variation between two treatments (water-stressed (ws) and well watered (ww) of the number of times a sieve element (Thin- or thick-walled sieve tube) is visited by *S. yakini.*

Treatment	SE	TWSE
WS	21.0 (± 1.8257)	1.25 (± 0.9574)
WW	22.5 (± 3.6968)	1.00 (± 0.8165)
P- value	0.49 (NS)	0.71 (NS)

Values are means of 5 replicates.

SE = Thin-walled sieve tubes, TWSE = Thick-walled sieve tubes, WS= water-stressed, WW= well watered. Level of significance: * $P<0.05$, ** $P<0.01$, *** $P<0.005$.

Table 3.7. Origin and distribution of stylets and stylet tracks/sheaths (probes) of *Sitobion yakini* after penetration of the leaf blades of barley

Condition	S & T	Source	Termination	WS	%	WW	%
			SE		**61**		
		adaxial		51		20	22
			TWSE	5	100	0	0
Sub totals				56		20	
			SE		**31**		
		abaxial		33		70	78
			TWSE	0	0	4	100
Sub totals				33		74	
TOTALS				89		94	

Note: Based on examination of 2000 serial sections.

S = stylets; T = tracks; WS = water stressed; WW = well watered.

Figs. 3.11 to 3.14 have been included to illustrate aspects of aphid feeding on water-stressed barley leaves.

Figure 3.11 to 3.14 illustrate the presence of unoccluded maxillary stylet tips (arrow) inside the thin-walled sieve tubes. In Fig. 3.11 the aphid probed the leaf (exposed to water stress) from the adaxial surface and terminated in a thin-walled sieve tube of a small bundle. The bundle sheath cell, mestome sheath cell and parenchyma cell were penetrated by the stylets on their way to the thin-walled sieve tube. Note that the maxillary stylet tips are separated and free of salivary material suggesting that the aphid was feeding from a functional sieve tube. Figure 3.12 illustrates a multiprobe, where the aphid unsuccessfully probed for a feeding site, then successfully probed the phloem. This probe originated from the abaxial surface of the leaf, the aphid first bypassed the vascular bundle traversing several bundle sheath cells to the left. After partly withdrawing the stylets, the aphid then penetrated several bundle sheath cells, to the right before terminating in a thin-walled sieve tube. The stylet tips (arrow) can be clearly seen inside the sieve tube. Although penetration of the xylem was largely intercellular, occasionally vessels and tracheary elements were penetrated (e.g., the vessel (T) in Fig. 3.12).

Figs. 3.13 and 3.14 show penetration of the thin-walled sieve tubes of an intermediate and small vascular bundles, respectively. The maxillary stylet tips are separated and free of any salivary material, again suggesting that the aphid was feeding. In Fig. 3.14 the aphid stylet pathway between the bundle sheath cells and mestome cells was intercellular.

Figure 3.11-3.14 show penetration of vascular tissue by *S. yakini*. Scale bars bars = 10µm. Open circles represent thin-walled sieve tubes.

Fig. 3.11. Shows the penetration of an intermediate bundle initiated from the adaxial surface of the leaf. The aphid stylet tips (unlabelled arrow) terminate inside a thin-walled sieve tube and are apparently free of any salivary material. The aphid sequentially probed a bundle sheath (BS), mestome sheath (MS) and vascular parenchyma (VP) cell before terminating in a thin-walled sieve tube.

Fig. 3.12. Here the aphid probed a large vascular bundle from the abaxial leaf surface. The aphid first penetrated a vacular parenchyma cell (VP) and then successively penetrated several thin-walled sieve tubes before terminating in one. Lack of salivary material suggests that the aphid was feeding from this functional sieve tube.

Fig. 3.13. Shows lateral penetration of an intermediate vascular bundle. The stylet tips are open and lodged within a thin-walled sieve tube. Note that the maxillary stylet tips are separated and free of salivary material, suggesting that the aphid was feeding.

Fig. 3.14. Shows penetration of a small bundle. The pathway of stylets from mesophyll through bundle sheath and mestome is intercellular. The aphid then penetrated parenchyma cells inter and intracellularly, before terminating in a thin-walled sieve tube.

Clearly, there seems to be no significant differences between probes of well-watered (Figs. 3.5-3.10) and water stressed plants (Figs. 3.11-3.14). In some instances there seem to be a bit more undirected probing (see Fig. 3.12 for example) than encountered in the water stressed material, but unfortunately insufficient data relating to direct probes or indirect probes was accumulated during the course of this study, to warrant any speculation.

As such, the only conclusion that can be safely made is that *S. yakini* managed to find a reasonably rewarding source of food when faced with a water stressed barley source.

3.5 Ultrastructure of penetrated vascular tissue.

Although light microscopy has been shown to provide an accurate and useful picture of the point of termination of the aphid stylets (Botha, Evert and Walmsley 1975a; Botha and Mabindisa 1977; Botha, Malcolm and Evert 1977; Botha and Evert 1978, Matsiliza and Botha 2000), several electron microscope studies such as those of Evert *et al*. (1973), Spiller, Kimmins and Llewellyn (1985) Kimmins (1986) and Tjallingii and Hogen Esch (1993) clearly show that electron microscopy provides a greater detail and resolution of the pathway followed by stylets. The use of electron microscopy is therefore essential when determining inter and intracellular penetration.

Evert *et al*. (1973) described the pathway of aphid stylets through the epidermis, mesophyll, and vascular tissue of barley. The entrance of the stylets through the epidermis and mesophyll was largely intercellular, the pathway apparently becoming intracellular only once inside the vascular bundle. The present transmission electron microscope study showed three types of penetration; intercellular (Fig. 3.15), intracellular and “intermediate” (Fig. 3.16) route with the saliva sheath between cell wall and protoplast. Figure 3.15 shows the intercellular passage of stylets between mesophyll cells. The stylets have collapsed under the beam, but salivary sheath material (SS) is visible between two mesophyll cells. The mesophyll cell to the left is partly collapsed and shows signs of membrane damage, possibly due to the aphid’s penetration and probing, or due to damage during specimen preparation. Figure 3.16 shows an example of an “intermediate” penetration. Here, the aphid has penetrated the cell wall, but the extruded salivary sheath material (SS) lies between the cell wall (CW) and the plasma membrane (PL). Little evidence could be found to show cell damage under these circumstances. The nucleus (N) adjacent to the sheath intrusion appears normal, as do organelles in the surrounding cytoplasm.

As mentioned there was evidence of damage of some mesophyll cells which could be attributed to intruding salivary material or rupture of the cell wall and subsequent loss of cell contents.

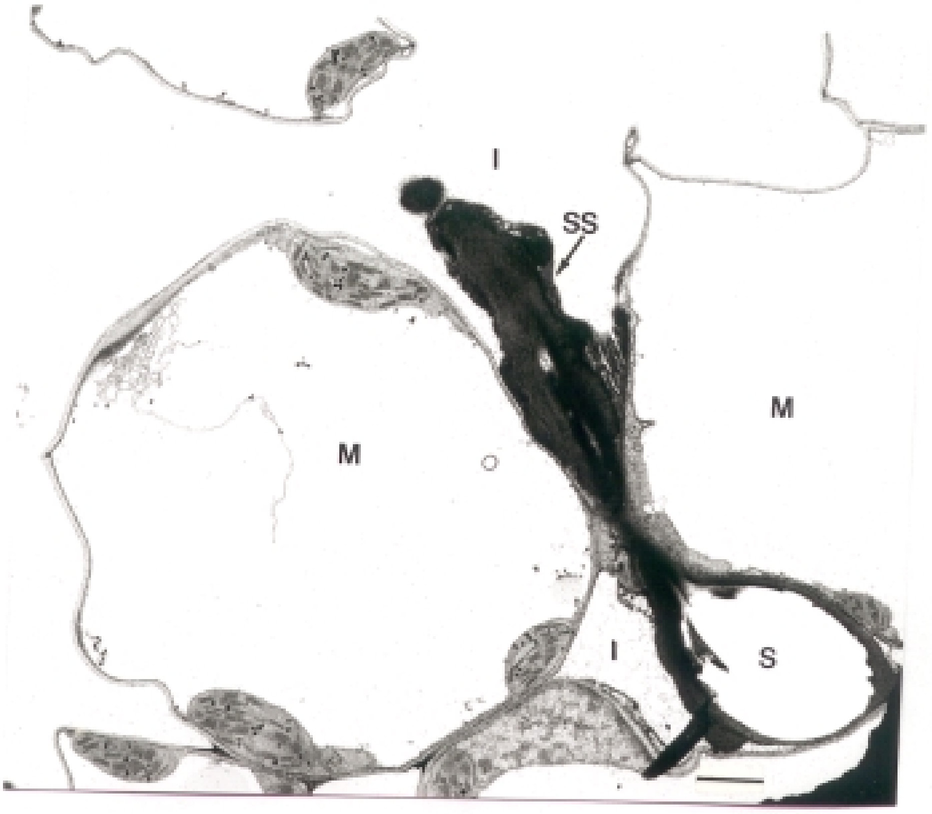

Fig. 3.15 Transverse section of maxillary stylets (S) (disintegrated) and stylet sheath (SS) in an intercellular space (I) between to mesophyll cells (M). Bar = 2µm

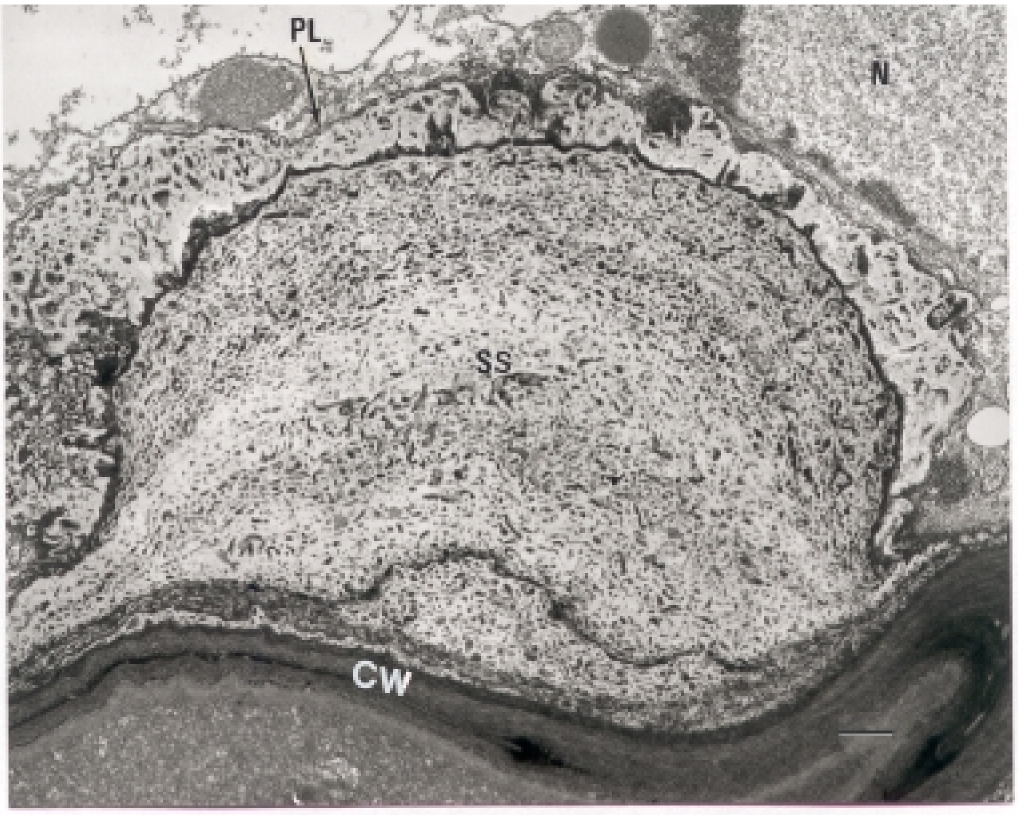

Fig. 3.16 Mesophyll cell with salivary sheath between the cell wall (CW) and plasmalemma (PL). The plasmalemma and protoplast have been pushed by the sheath material. Bar = 500nm

chapter 4: DISCUSSION

The data presented in Tables 3.1 and 3.5 support the hypothesis that *S.yakini* feeds preferentially on the thin-walled sieve tubes of barley plants grown under normal conditions and those exposed to a physiological droughting water stress. As with well-watered barley plants, most stylets and stylet track encountered in water-stressed plant material terminated in thin-walled sieve tubes. Of a total of 317 stylets and stylet tracks encountered in non-stressed barley plant material, 293 terminated in thin-walled sieve tubes (see table 3.1). Furthermore, 84 of 89 stylets and stylet tracks encountered in water stressed plant material and 90 of 94 encountered in well-watered plant material, terminated in thin-walled sieve tubes (see table 3.5). This data clearly shows that water stress did not affect the feeding pattern of *S.yakini* observed in barley plants grown under normal conditions (see Tab. 3.1 and also Matsiliza and Botha 2000).

The data presented in chapter 3 clearly support the hypothesis that the thin-walled sieve tubes are more attractive to *S. yakini* as a feeding site, in that 94% of stylets and 92% of stylet tracks, were seen to terminate in the thin-walled sieve tubes of the longitudinal veins in mature barley leaves. Under unstressed as well as water-stressed conditions, by far the majority of tracks and stylets (63%) were associated with the small longitudinal vascular bundles. Significantly nearly all probes terminated at or in thin-walled sieve tubes in the small veins, and in all only 0.5% of the probes terminated at or in thick-walled sieve tubes in intermediate (0.2%) and large (0.3%) longitudinal veins.

The data suggest that the preference for smaller veins is likely related to both the quality and quantity of the food (for example the total available soluble sugars, carbohydrates and proteins). Altus and Canny (1982) and Evert, Russin and Botha

(1996) suggested that these veins were involved in the assimilate loading process, and that the large bundles are primarily involved in longitudinal transport. If the small bundles are implicated in loading then clearly the closer the stylets are to the site of vein loading the higher both the quality and quantity of the food available to them should be. Equally so, osmotic dilution of the sieve-tube sap is likely further away from such sites (Milburn 1972, cited in Botha and Evert, 1978). The preponderance of stylets and tips associated with the thin-walled sieve tubes of small longitudinal bundles suggests potentially higher osmotic potentials within these veins.

Gibson (1972) and Elliot and Hodgson (1996) have suggested that vein-selection by aphids can be affected by several factors. First, the fact that the phloem of major veins is situated deeper within the leaf than is the case in minor veins, means that the aphid will expend more energy and time to reach its objective in sieve tubes. Feeding on minor veins means a shorter pathway to the functional sieve tubes. Secondly, in many plants the larger veins are associated with thick mestome or sclerenchyma sheath surrounding the vascular bundles, while smaller veins usually do not. Feeding on minor veins should therefore be easier for the aphid. Sclerenchyma girders are associated with large vascular bundles of barley leaves, and are found on the adaxial and abaxial side of the bundle. They do not surround the bundle. *S. yakini* has been found to approach the bundle obliquely and not directly from above, it seems quite possible for the aphid to avoid this structure obstacle and the position of sclerenchyma should not therefore impede the aphid’s probings.

By feeding on the minor, rather than major veins, the aphid has the advantage of a ‘shorter’ pathway to sieve tubes, less sclerenchyma to impede the passage of its stylets, and also a food supply which arguably is richer in both sugars and proteins.

Even though there was a 100% strike rate for thin-walled sieve tubes in small vascular bundles in both well-watered and water stressed plants, one cannot rule out the possibility that the transport veins (i.e., the large and intermediate veins) may be less “palatable” (i.e. contained a lower proportion of some highly desirable compound) to the aphid.

Of interest too was the fact that the aphids probed preferentially from the abaxial surface of the leaf in plants grown under normal conditions and from adaxial surface in those exposed to physiological stress. *Rhopalosiphum maidis* reportedly fed preferentially from the adaxial surface of the leaf in barley in the study reported by Evert *et.al*. (1973). However, the choice of penetration location exhibited by *S. yakini* may be due to the aphid simply preferring to feed from the abaxial surface rather than be exposed to potential predators if it fed on the adaxial leaf surface. The preference of the adaxial surface in stressed plants could simply be because the leaves were curled upward and thus feeding on the adaxial surface offered more protection than the now exposed abaxial surface would have done. Thus, the need for protection from potential predators and possibly to avoid being dislodged, could therefore account for the distribution of *S. yakini* on the abaxial surface in non-stressed plants and adaxial surface in water-stressed plants.

Several explanations have been put forward for the predominance of intercellular penetration of tissues by aphid stylets (see Evert *et al.* 1968 and literature cited therin). Firstly, that the intercellular course offers the line of least resistance. Secondly, that the cell wall offers support to the stylets. Thirdly, that smaller aphids are unable to penetrate the tissue by pressure alone and that their saliva is not potent enough to enable them to take a straight course to their objective. From the present investigation, the stylets of *S. yakini* (which is a fairly small aphid species) follow an apparently intercellular (from epidermis through the mesophyll) and then intracellular (near and inside the bundle) pathways.

While some workers have suggested that the aphids and other suctorial insects they have studied find their objectives by trial and error (Evert *et al*. 1968 and literature cited therein, Botha *et al.* 1975a,b), others have reported evidence which suggests that the aphid's stylets do not enter the tissues haphazardly, but are directed to their objective with marked precision (Chatters and Schlehuber, 1951 cited in Evert *et al*. 1968). Based on my observations, the aphid *Sitobion yakini* appears at times to locate the phloem by chance (Fig. 3.6,3.8,3.9, 3.10 and 3.12) and at other times with some degree of precision (Fig. 3.5, 3.7, 3.11, 3.13 and 3.14). However, it would seem that precise location may be more frequent, as branching of tracks, suggestive of trial and error, was relatively infrequent. It is not possible to rule out the probability that penetration releases pressure within the sieve tubes, and as such leads to decreased flow rate, at which point the aphid could withdraw its stylets, to look for a new feeding site. Quite possibly, multiple probes could indicate a long-term feeding pattern.

Prado and Tjallingii (1994) reported that during ingestion of phloem sap, the stylet tips of aphids projected beyond the salivary sheath which terminates at the cell wall, as the salivary sheath does not reach the sieve element. Several other authors also demonstrated that the maxillary stylet tips (of other aphids) projected beyond the salivary sheath during penetration (see Evert *et al.* 1968; Botha *et al.* 1975a,b; Botha, Malcom and Evert 1977; Botha and Mabindisa 1977; Botha and Evert 1978). In this study, it has been demonstrated that the maxillary stylet tips of *S. yakini* project beyond the salivary sheath in most cases and are open within the sieve tube being tapped. It would seem then, that the presence of open stylet tips and lack of associated salivary sheath material within the sieve elements, still constitutes sufficient evidence for such sieve elements to be identified as functional.

The effects of penetration and feeding of aphids and other suctorial insects upon host tissues have been reported by many investigators (see Evert *et al.* 1968; Evert, Eschrich, Eichhorn and Limbach 1973; Brzezina, Spiller and Llewellyn 1986). The effects range in severity from no apparent injury in some cases, to widespread disorganisation of cells in others. In this study, few cells appeared damaged due to stylet penetration at the light and transmission electron microscope level. However, more detailed studies are necessary to confirm this.

A question arising from the present study is why do the aphids feed almost exclusively on the thin-walled sieve tubes? It can be argued that structure or composition of the walls of the thick-walled sieve tubes makes it more difficult for the aphid to penetrate than that of the thin-walled sieve tubes. However, it must be born in mind that aphids have been found to penetrate or probe the bark of trees (see Evert *et al.* 1968) and penetrate long distances both intercellularly and intracellularly through xylem (Botha *et al.* 1975a) in search of internal phloem in stems of *Gomphocarpus physocapus*.

However, it is more plausible that the thin-walled sieve tubes contain some substance or substances which are either lacking in the thick-walled sieve tubes, or present in lesser amounts and that such substances are highly desirable to *S. yakini* within the sieve tubes of barley. Alternatively, perhaps greater quantities of assimilates are transported in the thin-walled sieve tubes than in the thick-walled sieve tubes and the aphids are subsequently drawn to higher sucrose concentration in these sieve tubes. The earlier formation and quantitative dominance of the thin-walled sieve tubes over thick-walled sieve tubes lends support to the idea that more assimilates are translocated in thin- than in the thick-walled sieve tubes.

Another consideration is that there could be resistance factors, which could possibly render the thick-walled sieve tubes unsuitable, or normally unpalatable to the aphid. However, if this was indeed the case, then one would have expected to see many more unsuccessful probes in the vicinity of the thick-walled sieve tubes in all bundle sizes than reported here. As this was not observed, the only conclusion one can come up with is that the thick walled sieve tubes do not possess these specific resistance factors.

Careful attention needs to be devoted to possible resistance factors. Two reasons are suggested in the literature which result in unsuitability of certain plants.These are chemical deterrence mechanism and mechanical interference (Caillaud and Niemeyer 1996). Possible chemical deterrence mechanisms in the phloem could include the occurrence of a feeding deterrent or the absence of a feeding stimulant, or a cardiac glycoside as was reported to exist in Asclepiadaceae and Apocynaceae which render the aphid, *Aphis nerii* immune to attack by predators (see Botha, Malcom and Evert 1977). These plant-derived toxic steroids (cardiac glycosides) are synthesised by *Apocynales* as an effective defence strategy against herbivores. The protective efficacy of a single cardiac glycoside in *Aphis nerii* fed on *Gomphocarpus physiocarpus* was indicated by the death of a dwarf chamaeleon which was fed about 200 *Aphis nerii* (Malcom 1976). A mechanical mechanism could involve the activation of the phloem sealing system of the plant which would result in the blocking of sieve elements at the sieve plates by callose or alternatively, in the plugging of the aphid’s stylets, thus reducing sap availability for ingestion. In addition, resistance factors have been shown to be located in the sieve elements in wheat which are able to inhibit the ingestion of sap by the aphid *Sitobion avenae* (Caillaud, Pierre, Chaubet and Di Pietro 1995).

Clearly, aphids select their feeding sites according to the quality and quantity of the food they yield, so feeding pattern may well be dictated by localisation of the phloem or of the sieve tubes within which the aphid will have access to both the quality and quantity of food required (soluble sugars, carbohydrates and proteins) where they also obtain other substances desirable or other essential food substances for them. Similar studies on phloem using aphids have also demonstrated a similar feeding strategy (Botha *et al*. 1975a, Botha and Evert 1978).

It is clear from past studies that thick-walled sieve tubes may be involved in the retrieval of photosynthates from the transpiration stream and/ or apoplast in grasses (see Fritz , Evert and Heyser 1983). Other studies (see Evert *et al.* 1996) suggest that the small veins in leaf blades of barley are involved in assimilate uptake and thus must have high solute concentrations which in this study, is confirmed as *S. yakini's* preferential feeding site, and then that thin-walled sieve tubes are preferred over the thick-walled sieve tubes. Whether it is sucrose or some other substance which is the attractant, remains to be determined.

4.1 Conclusion

This study shows unequivocally that *S.yakini* feeds preferentially from thin-walled sieve tubes of small longitudinal veins of the barley plant. The relatively low percentage of stylets and stylet tracks seen terminating at the thick-walled sieve tubes, clearly indicate that the thick-walled sieve tube is an unsuitable feeding site, or perhaps it does not contain enough of a particular substance (whether it is sucrose or not remains to be determined). Alternatively, the thick-walled sieve tubes may not have a sufficiently high functional osmotic potential, to allow feeding by *S.yakini* from them. Something is obviously missing from the ingredients required to make the thick-walled sieve tubes attractive to *S. yakini* with respect to providing an adequate food source.

Future research should focus on other potential stress inductants, such as elevated CO_2, high temperature and high light effects, to determine if these individually, or in combination, change or influence the feeding pattern of *S. yakini*. Callose formation in the phloem requires examination as well. Additionally a wider range of suitable aphids should be sought, to see if perhaps one or more species can be induced to feed from the thick-walled sieve tubes of barley.

Whilst the use of feeding aphids in this research project has provided information supporting the preferential feeding of the aphid on the thin-walled sieve tubes and adds to the small data pool previously available on the possible functions of thin- and thick-walled sieve tubes in grass leaves, it does not provide evidence for functionality of thick-walled sieve tubes in grasses. They remain as much of an enigma as they were when they were first reported 26 years ago by Kuo and O'Brien (1974).

However, much work remains to be done in order to determine the possible role of the thick-walled sieve tubes in grasses.

Suctorial insects such as aphids have a useful role in phloem structure/ function studies - without their we would be far less informed, and as yet, unable to speculate on the roles of thin- and thick-walled sieve tubes in nature.

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APPENDIX A

The full Long-Ashton nutrient solution used in this study as taken from Hewitt (1966).

Salt	Wt used (g)	V. stock Sol. (ml)	V. stock sol. dil. In 25 L (ml)	Conc. In final V. of 25 L (mM0
Macronutrient				
KNO_3	101	500	25	2
K_2SO_4	43	500	25	1
$Ca(NO_3)_2$	164	500	25	4
$CaCl_2$	111	500	25	4
$MgSO_47H_20$	92	500	25	1.5

$NaH_2PO_4.2H_2O$	104	500	25	4
Micronutrient				
$MnSO_4.4H_2O$	11.20	500	2.5	0.02
$CuSO_4.5H_2O$	1.25	500	2.5	0.002
$ZnSO_4.7H_2O$	1.45	500	2.5	0.002
H_3BO_3	15.50	500	2.5	0.05
$Na_2Mo_4.2H_2O$	0.605	500	2.5	0.0005
NaCl	29.30	500	2.5	0.1
Fe-Citrate (3H2O)	29.90	500	2.5	0.6

APPENDIX B

Table 1.: Alcohol dehydration series (from Botha, 1994)

	Step	Time in hours
1.	FAA	24h
2.	50% EtOH	12h
3.	70% EtOH	12h
4.	35% Butyl-alcohol	12h
5.	55% Butyl-alcohol	12h
6.	75% Butyl-alcohol	12h
7.	100% Butyl-alcohol	3 changes of 12 hours each
8.	100% n-Butanol (liqiud parrafin)	12h

Table 2: Composition of F.A.A.

Ethyl alcohol	50 cc
Glacial acetic acid	5 cc
Formaldehyde (37-40 %)	10 cc
Water	35 cc

Table 3.: Staining series (from Botha, 1994)

	Stain	Time
1.	Xylol	3 min
2.	Xylol	3 min
3.	Xylol	3 min
4.	Xylol : Alc. 50 : 50	3 min
5.	Abs. Alc.	2 min
6.	90% Alc.	2 min
7.	70% Alc.	2 min
8.	50% Alc.	2 min
9.	Safranin	24 hours
10.	70% Alc.	2 min
11.	90% Alc.	2 min
12.	95% Alc. + picric acid	dip drain
13.	95% Alc.= NH_3	dip drain
14.	Abs. Alc.	2 min
15.	Abs. Alc.	2 min
16.	Fast green	1 min
17.	clove oil	dip drain
18.	clearer	dip drain
19.	Xylol	2 min
20.	Xylol	2 min
21.	Xylol	max. 5-6 min

Table 5: T.B.A (Tertiary butyl alcohol)series.

T.B.A.	95% ethyl alcohol	Absolute ethyl alcohol	T.B.A.	Water	Paraffin oil
1	50	0	10	40	0
2	50	0	25	30	0
3	50	0	35	15	0
4	50	0	50	0	0
5	0	25	75	0	0
6	0	0	50	0	50
7	0	0	100	0	0