MECHANISM OF VECTOR RESISTANCE IN GROUNDNUT TO CONTROL *GROUNDNUT ROSETTE VIRUS* DISEASE IN SUB-SAHARAN AFRICA

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And so it went, they had come but some had gone: A mouse, a virus but Dudley he prevailed. Thank you for managing my computer-related pests. John and Nick, Lawrence and Sue, Dawn, Kate, Sandra, Don and Gay; the corridor on the second floor: I have been knocking on each door for questions or some jokes, a problem or something more. And there is Mark and Margaret, Chanban, Nathalie and Michelle downstairs. To all of you, thank you for your inputs and assistance.

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This thesis is dedicated to my mother 04 October 1950– 05 January 2000

It hurts us bad
To loose the one that we most love
We all move on
A little push a little shove

Three years ago
You moved away too far to be
I heart the news
The anger came the guilt to see

Now rest in peace
While words are still so hard to say
We all move on
and think of you this very day

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ABSTRACT

Analysis of biological performance parameters of *Aphis craccivora* on groundnut variety ICG12991 in laboratory and field trials demonstrated that ICG12991 was resistant to the aphid vector of groundnut rosette diseases and that this resistance was stable over time and under high aphid pressure. Feeding experiments related slow population development and high aphid mortality on ICG12991 to an inhibition of phloem feeding from the sieve elements. Consequently, virus transmission of all three agents of rosette disease was almost totally absent even under very high pressure of viruliferous aphids. Further investigation of the underlying mechanism of resistance in ICG12991 showed that the resistance was induced by aphid probes rather than constitutive. The induced plant response was described as a hypersensitive response around the aphid feeding sites. Aphid-resistance and hypersensitivity were identified in at least 7 groundnut varieties and it is proposed that more varieties are likely to express these characteristics.

The results led to the recommendation to evaluate groundnut varieties for aphid resistance by evaluating aphid population development. The identification of vector resistance and its efficacy in reducing virus spread of rosette disease in the field has opened the way for novel approaches for groundnut disease management.

LIST OF ABBREVIATIONS

AA:	Adapted Aphid Population
AAP:	Acquisition Access Period
ACMV:	*African cassava mosaic virus*
AIDS:	Acquired Immune Deficiency Syndrome
ANOVA:	Analysis of Variance
B:	Bud of developing leaves
BMV:	*Brome mosaic virus*
BtMV:	*Sugar beet mosaic virus*
BYDV:	*Barley yellow dwarf virus*
CMD:	*Cassava mosaic virus* disease
CMV:	*Cucumber mosaic virus*
CP:	Coat Protein
D:	Nymphal Development time in days
DAI:	Days After Inoculation
DAP:	Days After Planting
DBH:	Dot Blot Hybridisation
DfID:	Department for International Development
EPG:	Electronic Penetration Graph
FAO:	Food and Agricultural Organisation
GIS:	Geographic Information System
GRAV:	*Groundnut rosette assistor virus*
GRD:	*Groundnut rosette virus* disease
GRV:	*Groundnut rosette virus*
HD:	Honeydew Deposit
HEFCE:	Higher Education Funding Council for England
HIV:	Human Immunodeficiency Virus
HR:	Hypersensitive Response
IAP:	Inoculation Access Period
ICRISAT:	International Crops Research Institute for the Semi-Arid Tropics

LIST OF ABBREVIATIONS CONTINUED

IPM:	Integrated Pest Management
M:	Molar
Md:	Number of offspring produced by one adult aphid in time D
MSV:	*Maize streak virus*
NARO:	National Agricultural Research Organisation
NGO:	Non Government Organisation
No:	Number
Np:	No penetration
NRI:	Natural Resources Institute
NRInt	Natural Resources International
ORF:	Open Reading Frame
P:	Petiole
Pd:	Potential drop
PLRV:	*Potato leaf roll virus*
PR:	Pathogen Related
PSE:	Phloem Sieve Element
RH:	Relative Humidity
Rm:	Intrinsic rate of increase
RNA:	Ribonucleic Acid
ROS:	Reactive Oxygen Species
RRA:	Rapid Rural Appraisal
RT-PCR:	Reverse Transcription-Polymerase Chain Reaction
S:	Main stem
SA:	Susceptible Aphid population
SAARI:	Serere Agricultural and Animal production Research Institute
SatRNA:	Satellite RNA
SB:	Side Branch
SCRI:	Scottish Crops Research Institute
S.E.:	Standard Error of the mean
SEM:	Scanning Electron Microscope

LIST OF ABBREVIATIONS CONTINUED

SQRT:	Square Root
SSA:	Sub-Saharan Africa
TAS-ELISA:	Triple Antibody Sandwich- Enzyme Linked Immunosorbent Assay
TYLCV:	*Tomato yellow leaf curl virus*
UK:	United Kingdom
UoG:	University of Greenwich
USAID:	United States Agency for International Development
UV:	Ultra Violet
USA:	United States of America

bp:	base pair
°C:	degrees Celsius
cm:	centimetre
diam:	diametre
div:	diverge
e.g.:	example given
g:	gram
h:	hour
Kg:	Kilogram
mg:	milligram
min:	minute
ml:	millilitre
mm:	millimetre
mV:	millivolt
µm:	micrometer
ppm:	parts per million
sec:	second
w/v:	weight per volume

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW ON GROUNDNUT PRODUCTION IN SUB-SAHARAN AFRICA

1.1. The project in the context of African agriculture

1.1.1. Characterisation of African agriculture

In sub-Saharan Africa (SSA), agriculture should be a driver of economic growth but public agricultural research in this region has long been hampered as a result of political instability and poor macroeconomic policies (Laker-Ojok, 1996). The result is often a vicious circle of low input/low productivity agriculture characterised by small-scale farmers cultivating land holdings of less than a hectare to a few hectares in area. The farmers' main objective is subsistence and the farms are situated in areas of low agricultural potential associated with unfavourable soil conditions, unpredictable rain patterns and limited opportunities to improve soil and water management (Chambers, 1990).

Agricultural production in Africa is adversely affected by pests such as insects and other invertebrates, pathogens, weeds, fungi and vertebrates (Oerke *et al.*, 1994). With urban and peri-urban populations rising, a declining rural population will have to increase food production. This process is seriously hampered by the spread of human diseases such as HIV/AIDS, which is infecting millions of people in Africa and causing serious labour shortages for both farm and domestic work, as well as human suffering (Annan, 2003).

In SSA, pest problems in agriculture are associated with efforts to intensify agricultural productivity in order to meet a growing demand for food and export income, and the accidental introduction of exotic pests and diseases (Waage, 1993). Pest control

strategies include biological control, indigenous methods of pest management, cultural control, use of resistant crop varieties, bio-pesticides, regulatory functions and plant quarantine. The selection and application of these tactics, singly or harmoniously coordinated into a management strategy that takes into account the interests of the producers, society and environment, is commonly known as Integrated Pest Management or IPM (Kogan, 1998). Pesticide use does not always meet this requirement and many smallholder farmers in Africa lack the resources to purchase chemicals. Furthermore, pesticides are ecologically disruptive (Greathead, 1986), are health hazards and eventually lose their effectiveness through the development of resistance. Nevertheless, in the developed world it is still the main tactic applied to control pests and diseases.

In contrast to the situation in Asia, IPM successes in SSA are limited and the main constraints are discussed in a range of papers (Goodell, 1984; Wearing, 1988; James, 1989; Maxwell, 1990; Schulten, 1990; Iles & Sweetmore, 1991; Glass, 1992). The report of an IPM workshop held in the Republic of Benin by non-government organisations (NGOs) in 1999 outlined more details on the important barriers to an effective incorporation of IPM in Africa (Assétou *et al.*, 1999). Apart from pests and diseases, these include:

1. Uncertain rainfall and poor soil fertility (Goodell, 1984).
2. Translation of IPM messages in a language and in packages that can be understood and applied by farmers (Goodell, 1984).
3. Lack of trained extension officers (Zethner, 1996) and transport to reach rural communities. In addition, IPM projects require managers who can organise, direct, co-ordinate and plan.
4. Low prioritisation by national governments, international donors and the private sector to support IPM.

In spite of these barriers, national and international collaborations have managed successful projects such as the management of *Cassava mosaic virus* disease (CMD) in Uganda. After it was reported that the disease had reached overwhelming proportions

in 1982, international collaborations led to a research programme in 1992. The likelihood of impact and adoption of the outputs were high because there was clear evidence of demand from farmers, politicians and economists. Also, resistant germplasm of cassava was available and could be developed into locally appropriate accessions by national research centres. Tolerant and resistant varieties were bulked up and distributed and more resources became available to understand what was driving the CMD epidemic. Other successful introductions of improved varieties, which increased yields dramatically for different crops, were cassava in Nigeria (Nweke *et al.*, 1994), maize in Central and West Africa (Manyong *et al.*, 1995) and sorghum, sweet potato, cowpea and maize in Mozambique.

1.1.2. The establishment of the research project and objectives

1.1.2.1. Identification of major groundnut production constraint

In Uganda, groundnut, *Arachis hypogaea* L., is the second most widely grown legume after common beans *Phaseolus vulgaris* L. and is mainly cultivated in the major production areas in the Eastern Region (see also 1.2.2.1). A Rapid Rural Appraisal (RRA), which is an assessment of farmers' circumstances and farming needs, was carried out in 1998 in the Teso farming system, which is a semi-arid production system in the north-eastern parts of Uganda. The RRA identified *Groundnut rosette virus* disease (GRD), an aphid-vectored virus disease, as a very important production constraint of groundnut. The assessment was organised by Ugandan national organisations in collaboration with the Department for International Development (DfID) of the UK Government and formed the basis of a DfID Crop Protection Programme funded project (R 7445) to develop and promote management strategies against groundnut rosette. Developing and screening drought and rosette resistant varieties and the improvement of groundnut seed production at the community level were high priority issues for future research because groundnut is an important cash crop, access to markets is good and returns for the local farmers are high. As well as screening varieties in the Teso system, it was decided that the mechanisms of resistance should also be explored as this would feed back into groundnut breeding and improvement programmes.

1.1.2.2. Summary of previous results leading to the research project

Varieties resistant to groundnut rosette disease were first identified in West Africa (Sauger & Cathérinet, 1954 a,b) and have been used in breeding programmes throughout SSA thereby contributing to the development of several disease-resistant varieties (Gibbons, 1977; Bocklee-Morvan, 1983). The major disadvantage of these resistant varieties, however, was that they required a long growing season of 150 to 180 days to attain maturity therefore making them susceptible to drought at the end of the rainy season. In 1982, the International Crop Research Institute in the Semi-Arid Tropics (ICRISAT), based in Malawi, established a regional groundnut improvement programme for Southern Africa to develop agronomically and commercially acceptable groundnut varieties with resistance to major diseases including rosette. A simple but effective field screening technique was developed to evaluate rosette-resistant germplasm, known as the infector row technique (Bock, 1987). The technique is based on the planting of rows of GRD infected plants at regular intervals between the plots of germplasm to be evaluated (Figure 1.1). The infected rows are manually infested with aphids which can acquire and transmit the virus agents of rosette disease to the surrounding plots. To date, thousands of germplasm lines have been screened to diversify the genetic base of rosette resistance and over 20 early-maturing (90-110 days) rosette resistant lines have been identified with excellent performance in on-farm trials in Malawi, Zambia, Mozambique and Uganda (Merwe van der & Subrahmanyam, 1997; Subrahmanyam, *et al.*, 1998). In these trials, farmers are encouraged to attend on-farm demonstrations to increase awareness and adoption of improved varieties and other crop management methods. They will also have the opportunity to evaluate the benefits of the improved methods and gain experience with the new technologies.

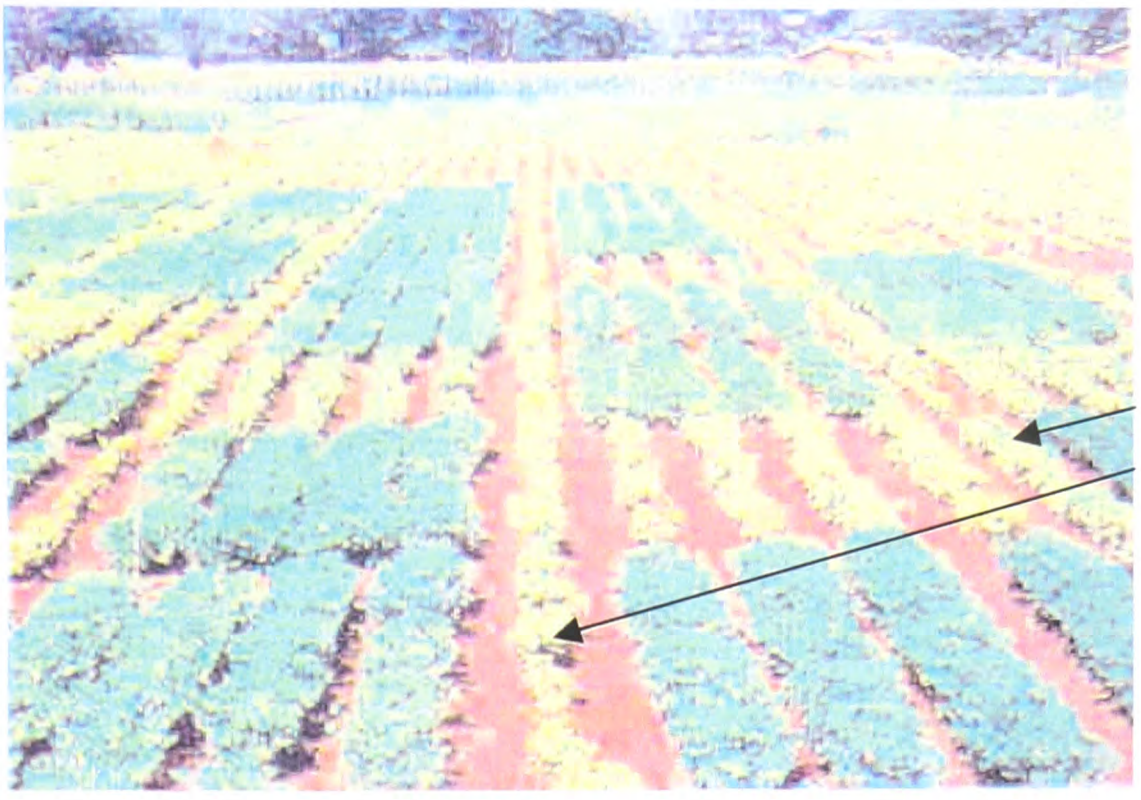


Figure 1 1: Screening groundnut varieties for resistance to *Groundnut rosette virus* disease using the infector row technique (Bock, 1987) at the International Centre for Research in the Semi-Arid Tropics, Chitedze, Malawi.

In 1997, several rosette-resistant varieties were identified using the infector row technique at ICRISAT-Malawi (Naidu *et al.*, 1999b). ICG12991 was of particular interest due to its agronomic traits, its high yielding potential and short maturation period (90-110 days). After recognition of the importance of introducing rosette-resistant germplasm as a tool to control rosette disease, preliminary research on its resistance mechanism was initiated. Grafting virus-infected scions of a susceptible variety JL24 to the healthy rootstocks of ICG12991 and *vice versa* resulted in new virus infected shoots indicating virus-susceptibility of ICG12991 to all virus agents of the disease complex (Figure 1.2) (Merwe van der *et al.*, 2001). Further characterization of rosette resistance in ICG12991 and its underlying mechanism formed the backbone of the work presented here of which the objectives are outlined in the following section (1.1.2.3).

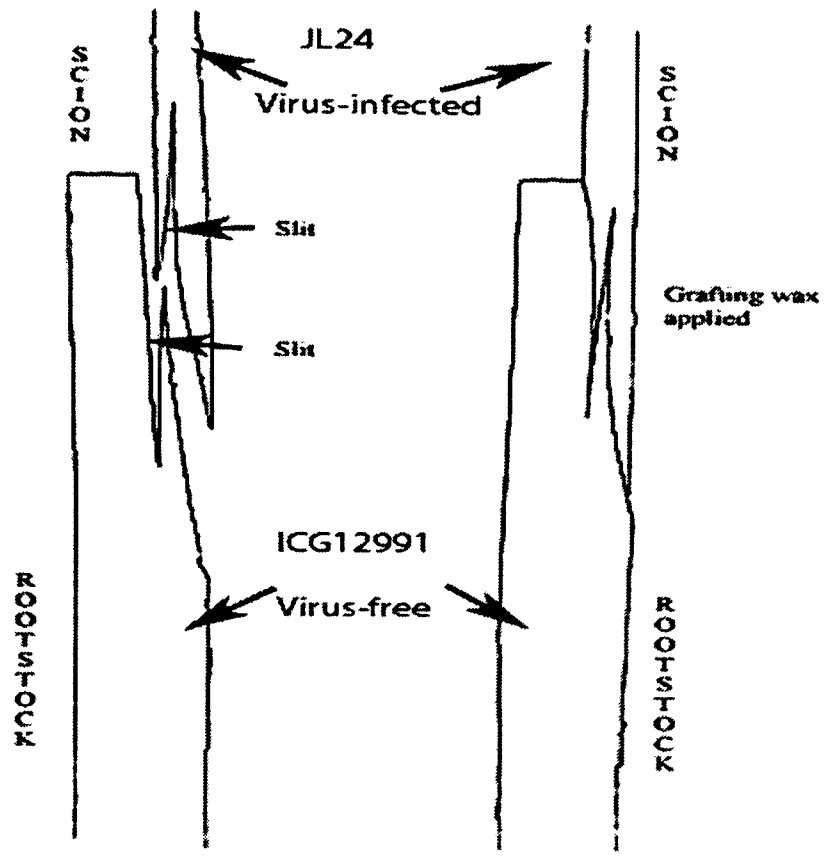


Figure 1 2: Illustration of the grafting technique in which virus-infected scions of a susceptible groundnut variety JL24 were grafted on healthy rootstocks of ICG12991. Emerging shoots from the rootstocks were virus-infected illustrating virus susceptibility in ICG12991.

1.1.2.3. Objectives

1. To evaluate vector resistance in selected groundnut varieties by investigating aspects of aphid host plant selection under controlled and field conditions (Chapters 2, 3).
2. To correlate the results on aphid resistance to vector transmission of the GRD virus agents (Chapter 4).
3. To describe the mechanism of resistance to the GRD complex in groundnut variety ICG12991 (Chapters 5, 6).
4. To investigate the durability of the resistance to vector and disease (Chapter 7).
5. To summarise and evaluate research outputs and discuss the potential of naturally occurring plant resistance in groundnut production (Chapter 8).

1.2. Literature review on groundnut cultivation in sub-Saharan Africa

1.2.1. History, origin and morphology of the groundnut crop

Arachis hypogaea L. is an annual legume (Fam. *Leguminosae*) native to the eastern slopes of the Andes in South America. The species was introduced to Africa, Asia and to the Pacific Islands and finally to the south-eastern United States by explorers and missionaries in the 16th century (Sinha & Bhagat, 1988). The first species of *Arachis*, described by Linneaus in 1753, was *Arachis hypogaea* (Figure 1.3). The genus is unusual amongst plants because fertilisation activates an intercalary meristem (a peg or gynophore) which grows geotropically from the ovary after fertilisation and carries the developing embryo into the soil (Darwin, 1880). The pod expands and the embryo grows rapidly to produce subterranean seeds, which are commonly known as groundnuts or peanuts. *A. hypogaea* and the wild relative, *Arachis monticola* L. have 4X genome and both are natural tetraploids (4n = 40) (Krapovickas, 1973; Gregory *et al.*, 1980). The species are self-pollinating, but out-crossing does occur in approximately 2.5% of the flowers (Norden, 1980).

1.2.2. Groundnut cultivation in sub-Saharan Africa

The five most important legumes produced in the tropics are the common bean (*Phaseolus vulgaris* L.), cowpea (*Vigna unguiculata* Walp.), groundnut (*Arachis hypogaea* L.), pigeon pea (*Cajanus cajan* L.) and soybean (*Glycine max* L.) (Duke, 1990). Together, these legumes provide much of the needed protein, vitamins and minerals to the subsistence farmers and the rural poor and, by fixing nitrogen, legumes improve soil fertility thereby increasing productivity of the cereal cropping systems (Naidu *et al.*, 1999b).

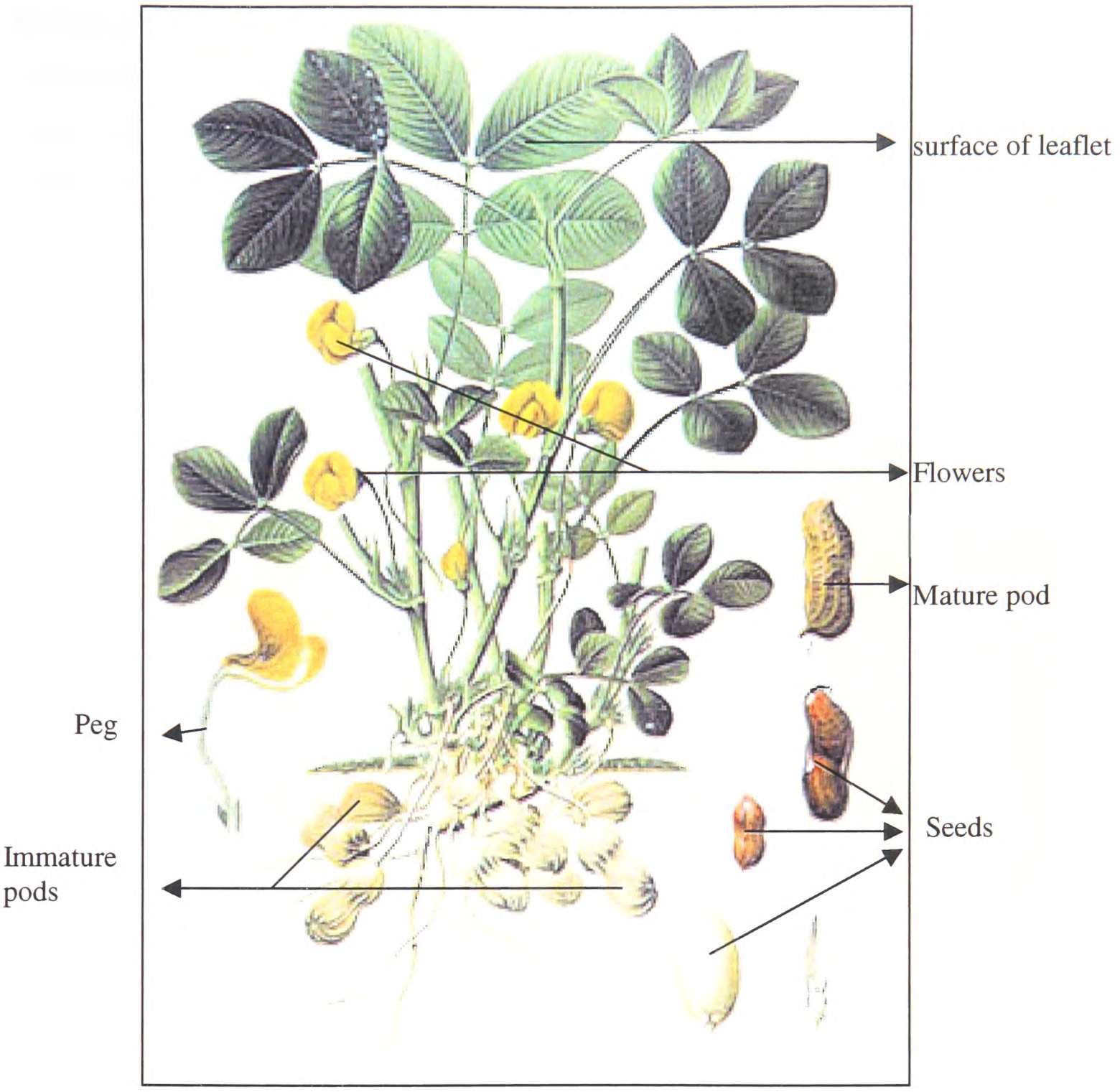


Figure 1 3: Morphology of *Arachis hypogaea* L.

(c) 1995-2002 Missouri Botanical Garden

http://ridgwaydb.mobot.org/mobot/rarebooks/

Groundnuts are grown in most countries of SSA as a subsistence crop under rain-fed conditions, either once (e.g. Central Malawi) or twice a year (e.g. Eastern Uganda), depending on rainfall patterns. In Central Malawi (Figure 1.4a), a single growing season varies in length from less than 120 days to over 210 days (November-December to April). In Uganda (Figure 1.4b), the first rains arrive in March-April and this growing season lasts until July. The second rainy season begins in September and continues till January.

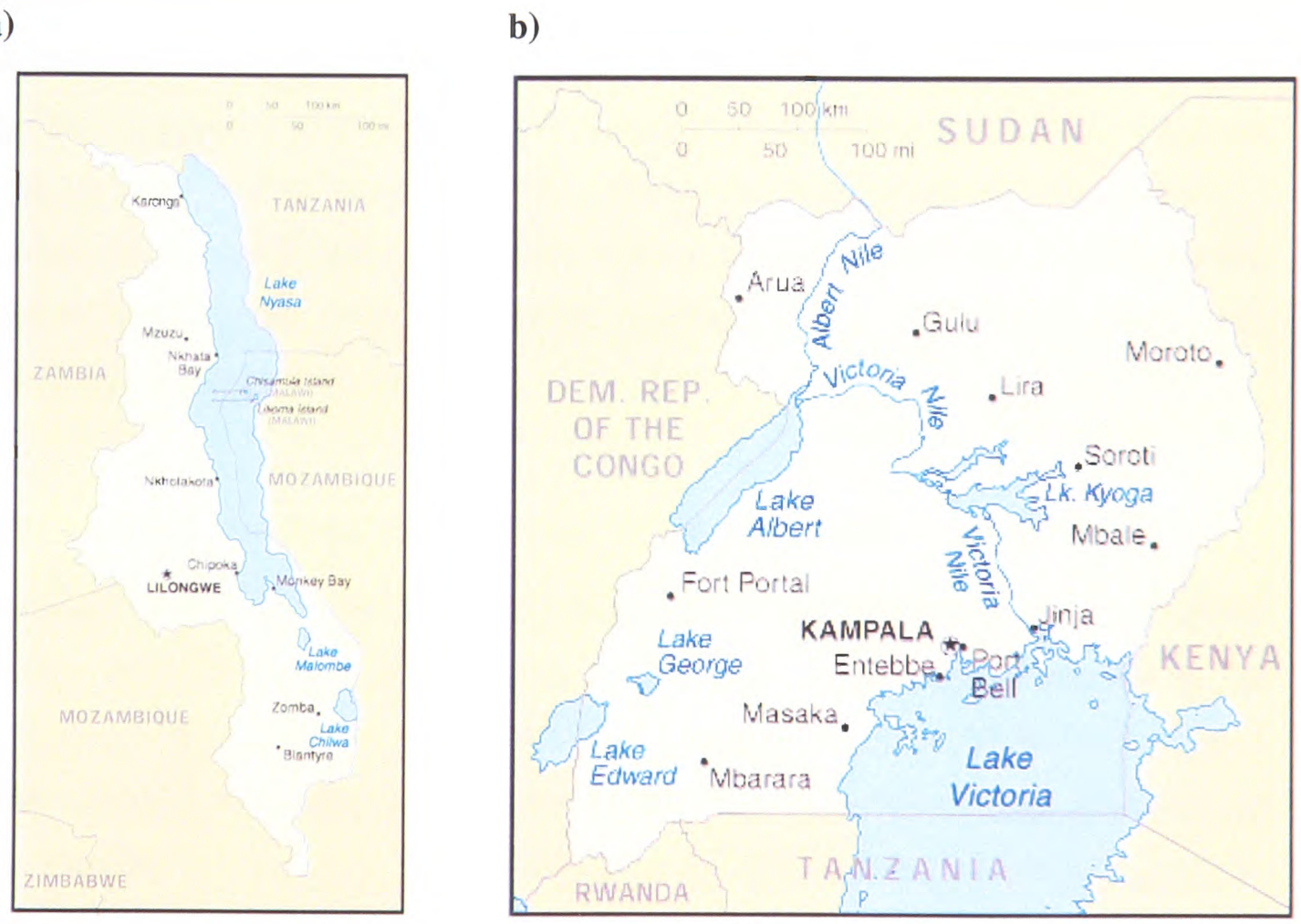


Figure 1 4: Geographical maps of the East-African countries, Malawi (a) and Uganda (b).

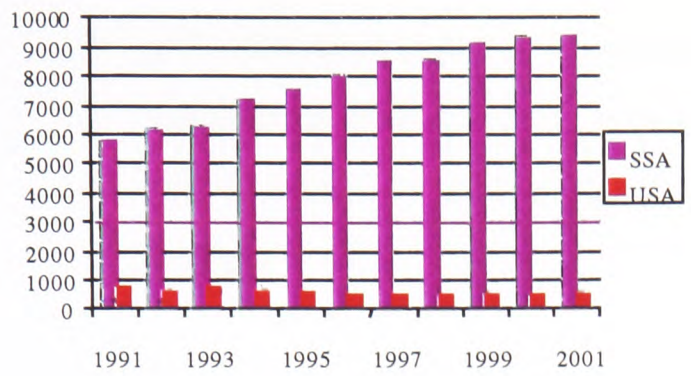
http://www.lib.utexas.edu/maps/africa.html

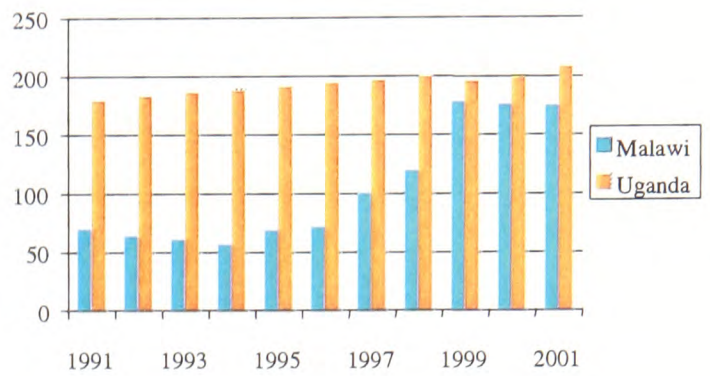
Groundnut is now cultivated in the semi-arid tropical and subtropical regions of nearly 100 countries on 6 continents between 40°N and 40°S (Naidu *et al.*, 1999b). It is mainly grown in Asia, especially India and China and other key zones include Southern and Western Africa. For the developed world, the United States of America are the leading producers of groundnuts (Wightman *et al.*, 1990) with yields per unit area tripling those of SSA (http://apps.FAO.org). Despite this disparity, the area harvested and the production of groundnuts in shell in SSA has almost doubled in the past 10 years from 6 x 10^6 ha with a production of 4800 x 10^9 tons in 1991 to 9.5 x 10^6 ha and a production of 8100 x 10^9 tons in 2001 (Figure 1.5). More details are provided for two countries of main interest in SSA: Malawi and Uganda (Figure 1.4). The increasing area harvested and production over the last 10 years reflect the growing importance of groundnut, especially in Malawi. Yields ranged from 400-900 kg/ha and are subject to a range of biotic and abiotic factors. The area harvested and production for the leading producers in the developed world (USA) slightly decreased within the 10-year period. However, yields in the USA were almost three times those from SSA, where yields were always less than 1000 kg/ha.

1.2.3. Major constraints in the groundnut production process

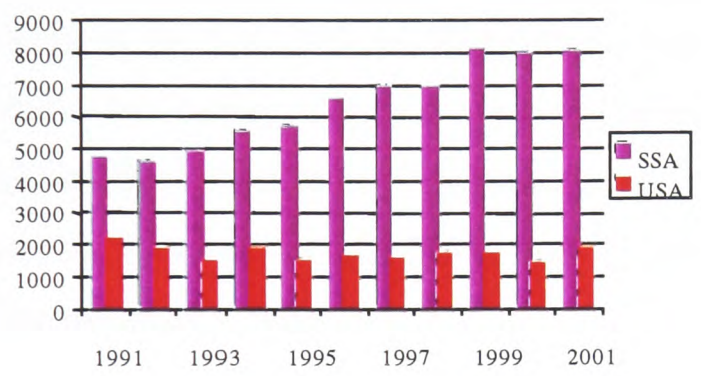
Constraints to groundnut production are many and varied both between and within the different states of SSA (Subrahmanyam *et al.*, 1997). Compared to the rest of the world, yields are low for a number of factors including inadequate rainfall, lack of good quality seed, labour, capital and/or information about improved cultural practices and most importantly, pests and diseases (Naidu *et al.*, 1998). Many insect pests, fungal, viral, nematode and bacterial diseases attack groundnut from germination to post-harvest but only a few of them are economically important (Subrahmanyam *et al.*, 1997). Insect pests of groundnut were first extensively reviewed by Feakin (1973) and Wightman and co-workers discussed specifically insect pests of groundnuts grown in the Semi-Arid Tropics (Wightman *et al.*, 1990).

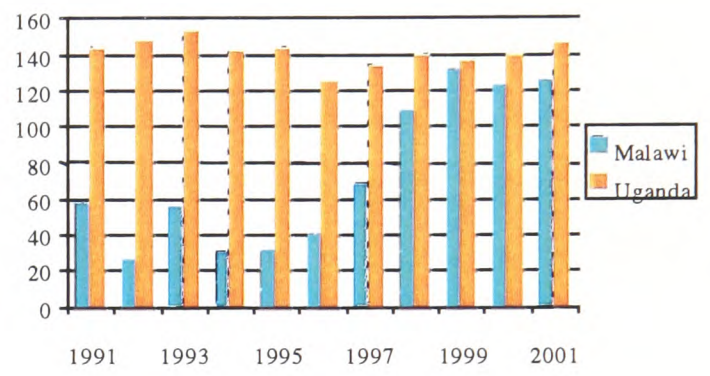
Area of harvest (1000 ha)



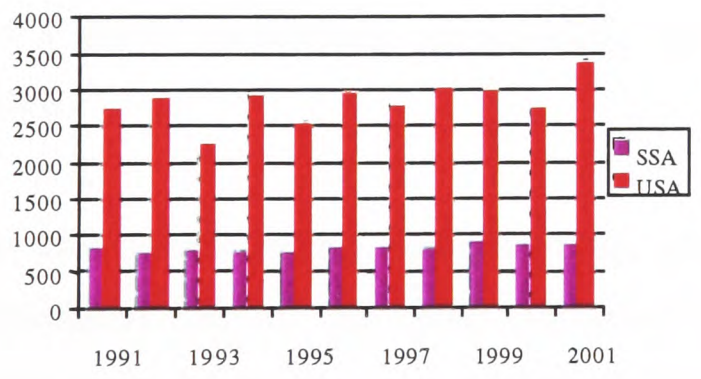


Production (10^3 tons)





Yield (Kg/ha)



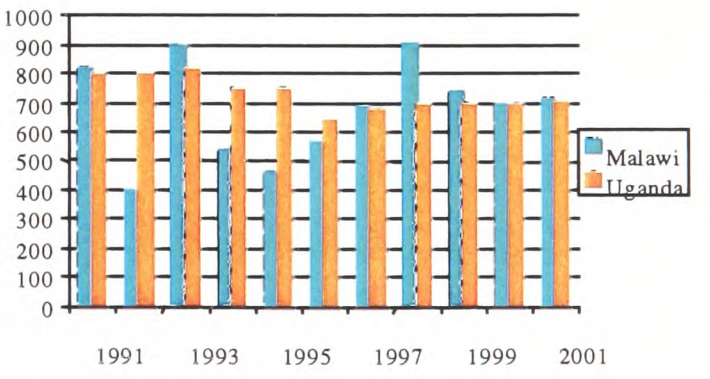


Figure 1 5: Area of harvest, production and yield of groundnuts in shell in sub-Saharan Africa, USA, Malawi and Uganda

http://apps.FAO.org

Four cohorts of **insects** attack groundnut:

1. non-viruliferous foliage feeders (e.g. grasshoppers, caterpillars)
2. soil- inhabiting insects (e.g. termites)
3. viruliferous foliage feeders (virus vectors, e.g. aphids)
4. post-harvest insects feeding on the harvested and stored pods (e.g. bruchid beetles)

Foliage feeders are often conspicuous in groundnut fields but apart from destruction by locust plagues there are not many reports of major yield losses due to acridids.

Termites were recognised as important pests of groundnut in Africa, especially those living in the soil such as *Microtermes* spp. and *Odontotermes* spp. They can kill the plants by boring into the root and felling the stems by chewing through a few millimetres of the stem (Wightman *et al.*, 1990), boring into the pods and removing the kernels (Johnson *et al.*, 1981) and by depleting the crop as it is drying in the field (Burrell *et al.*, 1965).

The virus-vectors such as various families of the Homoptera (aphids, whiteflies, leafhoppers and delphacid planthoppers) and especially the *Aphididae*, cause damage to the crop by their direct feeding behaviour but their pest status is mainly due to their role as a vector transmitting economically important viruses such as *Groundnut rosette virus*.

In post-harvest stores, females of the groundnut weevil (*Caryedon serratus*, Fam. Bruchidae, Olivier) can cause substantial losses by attaching their eggs to the pods. First instar larvae then burrow through the pod wall and the seed coat to feed on the cotyledons (Conway, 1975; Dick, 1987).

The most important **fungal diseases** include early leaf spot (*Cercospora arachidicola* Hori), late leaf spot (*Phaeoisariopsis personata* Berk. & Curt. Arx) and rust (*Pucchinia arachidis* Speg.) (Subrahmanyam, *et al.*, 1997). They are common in many production

systems and the predominance of each is largely dependent on climatic conditions. Other seed- and soil-borne fungi are widespread such as *Aspergillus niger* (van Tieghem); *A. flavus* (Link ex fr.); *Rhizoctonia solani* (Kuhn); *Macrophimina phaseolina* (Tassi.Goid); *Phytium* spp. and *Fusarium* spp. A lot of attention is given to aflatoxin contamination caused by *Aspergillus flavus*. In groundnut, it poses a serious threat to human and livestock health (Cole, 1991) because mycotoxins can accumulate in the groundnut crop during the entire crop growth period and during storage.

Important groundnut **viral diseases** include *Groundnut streak necrosis*, *Peanut clump virus* and *Groundnut rosette virus*. *Groundnut streak necrosis* is transmitted by *Thrips tabaci* and *Frankliniella occidentalis* (Thysanoptera) and also spreads through whitefly. Since the disease is seed-borne, farmers should also avoid using seeds from infected crops. In 2000, the disease devastated the largest single groundnut-growing area in the world (Ananthapur and Kurnool districts of Andhra Pradesh, India), causing crop losses of more than US$ 64 million and affecting the lives of more than half a million farmers. *Peanut clump virus* disease (PCD) is a major pest on groundnut in West Africa (Naidu *et al.*, 1997) and is mainly transmitted by the fungus *Polymyxa graminis*. *Groundnut rosette virus* disease (GRD) is the most important viral disease in SSA where it is endemic and transmitted by the aphid *Aphis craccivora* Koch (Homoptera, Aphididae) (Naidu *et al.*, 1999b). In 1975, an epidemic in northern Nigeria destroyed approximately 0.7 million ha of groundnuts, with an estimated loss of US $250 million (Yayock *et al.*, 1976). The most recent GRD epidemic (1994-1995) in Central Malawi destroyed the crop to such an extent that the total area of groundnut grown in Malawi fell from 89,000 ha in 1994-1995 to 69,000 ha in 1995-1996 (Anonymous, 1996). However, data about the area harvested vary according to the source. FAO estimates an area of 57,000 ha in 1994/1995 and 69,000 ha in 1995/1996 (Figure 1.5). Overall yield loss due to GRD in Africa was estimated at about US$ 156 million per annum.

1.2.4. Groundnut Rosette Disease Complex

The major biotic constraint in groundnut production in SSA is groundnut rosette disease (GRD), which was first reported in 1907 from Tanganyika (Zimmerman, 1907), now Tanzania. The disease has now been reported throughout other groundnut growing countries in SSA including Madagascar. Since GRD is endemic to SSA it is considered to be an example of the new-encounter phenomenon. This occurs when a crop has been introduced into a new geographical area and pests and/or pathogens that evolved with other host species attack the newly introduced crop (Buddenhagen & Ponti de, 1984).

More than 70 years ago H. H. Storey demonstrated that a virus, transmitted by the aphid *Aphis craccivora* Koch, in a persistent and circulative manner, caused the disease (Storey & Bottomley, 1928; Storey & Ryland, 1955; 1957). The ICRISAT scientists have continued the work on the aetiology of GRD and in the process have established excellent partnerships with advanced research institutes such as the Rothamsted Research, the Scottish Crops Research Institute (SCRI, Scotland) and the Natural Resources Institute (NRI, UK).

1.2.4.1. Groundnut rosette disease

By the early 1990s it was established that GRD had a complex aetiology involving three agents: *Groundnut rosette umbravirus* (GRV) (Murant *et al.*, 1995) and its satellite RNA (satRNA), (Blok, *et al.*, 1994) and *Groundnut rosette assistor virus* (GRAV), Fam. *Luteoviridae* (Murant, 1989). Single infections with either GRAV or GRV are symptomless or cause transient mild mottle symptoms. The rosette symptoms are largely due to satRNA (Murant, *et al.*, 1988), and variants of satRNA are responsible for different forms of the disease: 'chlorotic' rosette and 'green' rosette (Storey & Bottomley, 1928; Réal, 1955; Murant & Kumar, 1990) (Figure 1.6; Appendix 1). In nature, GRV and satRNA have always been found together. The three agents are dependent on each other and all play a crucial role in the biology and perpetuation of the disease (Naidu *et al.*, 1999a). The satRNA depends on GRV for replication in plant tissue and on GRAV for aphid transmission. In turn, the satRNA mediates the dependence of GRV on GRAV (Murant, 1990). GRAV forms a coat protein and the GRV RNA and satRNA can only be transmitted when packaged together in the coat protein of GRAV (Robinson

et al., 1999). Therefore the disease can only be acquired from the phloem. It has also been suggested that GRAV is dependent on GRV and satRNA, the symptom-causing agents. The symptoms might attract migrating aphids and subsequently contribute to the spread of the disease (Réal, 1955) (broken line) (Figure 1.7).

Figure 1 6: *Arachis hypogaea* showing the symptoms of chlorotic variant of groundnut rosette disease, which include severe stunting and chlorosis of the leaves

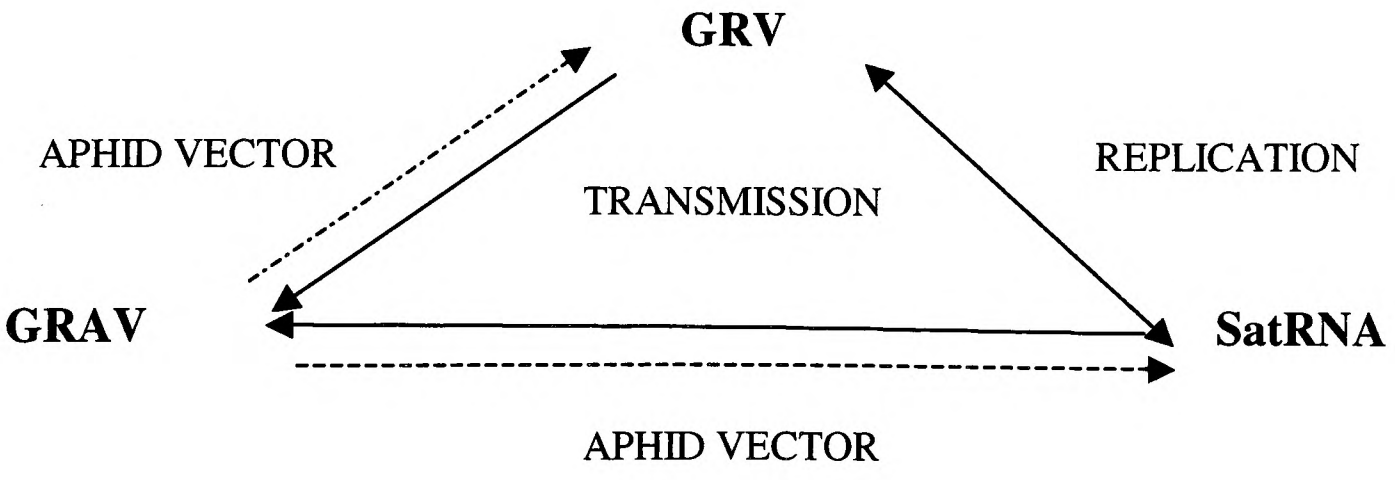


Figure 1 7: The interrelationships between the viral agents of groundnut rosette disease (GRV, satRNA and GRAV) and the aphid vector. Broken line is hypothetical, see text for explanation.

1.2.4.2. Aphid vector (*Aphis craccivora*, Koch)

H. H. Storey demonstrated in 1928 that the aphid *Aphis craccivora* Koch (Homoptera, Fam. *Aphididae*) (Figure 1.8a,b) is the major efficient vector of GRD in nature (Storey & Bottomley, 1928). A second aphid species, *Aphis gossypii* Glov., was reported to be a relatively inefficient vector (Adams & Farrell, 1967), but this has not been confirmed outside Malawi. *A. craccivora* infests many plant species in many families, but has a strong preference for members of the *Leguminosae*, which account for 47% of the known host species (Eastop, 1981). In a catalogue of aphids of SSA, Millar (1994) listed 142 plant species in 23 families as hosts of *A. craccivora*, of which 83 are in the *Leguminosae*. In the tropics, only females have been recorded and these reproduce parthenogenetically throughout the year. The nymphs develop through five instars in a short period (6 days) and the ability to reproduce rapidly and develop winged morphs (alatae; Figure 1.8a) at relatively low population densities makes it an opportunistic coloniser (Mayeux, 1984; Réal, 1953). The distribution of the aphid and the occurrence of GRD in the world are presented in Figure 1.9.

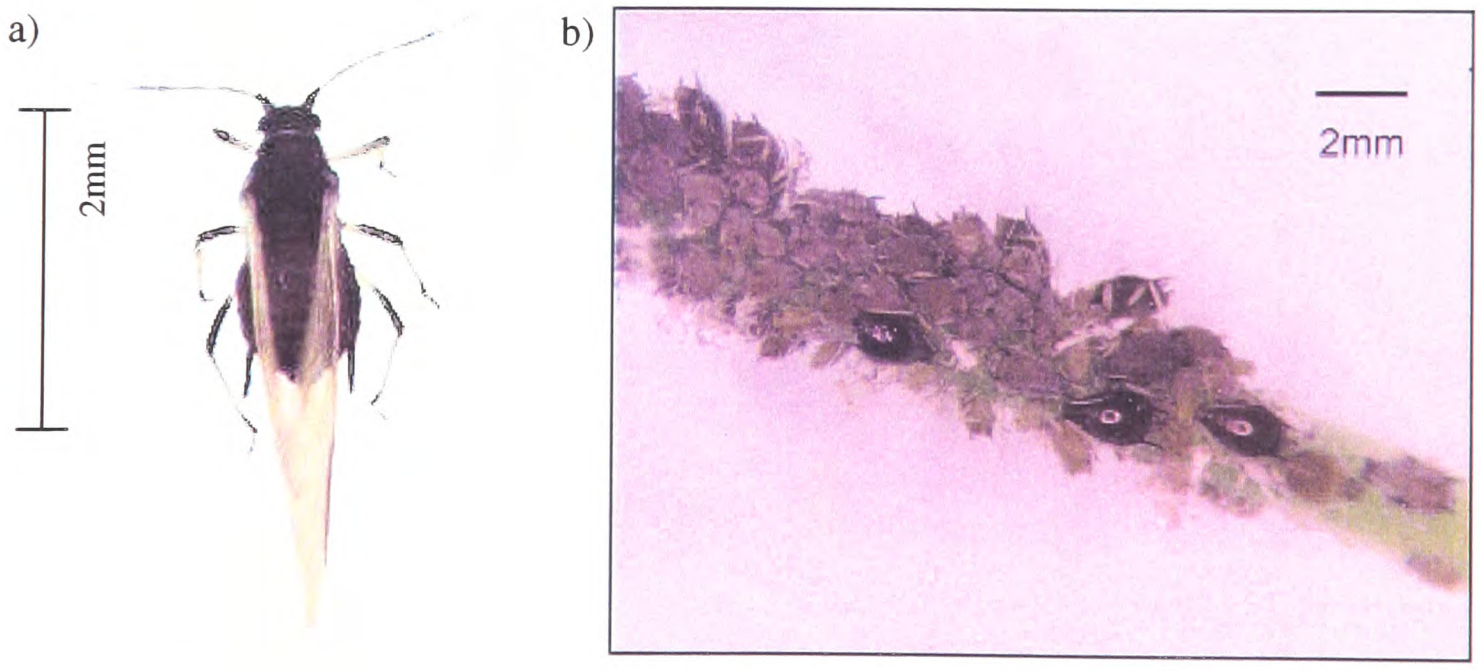


Figure 1 8: *Aphis craccivora* adult alate morph:
http://www.ento.csiro.au/aicn/systematic/c_980.html

Figure 1.8b: Colonies of immature and mature (shiny black) aphids on a groundnut stem.

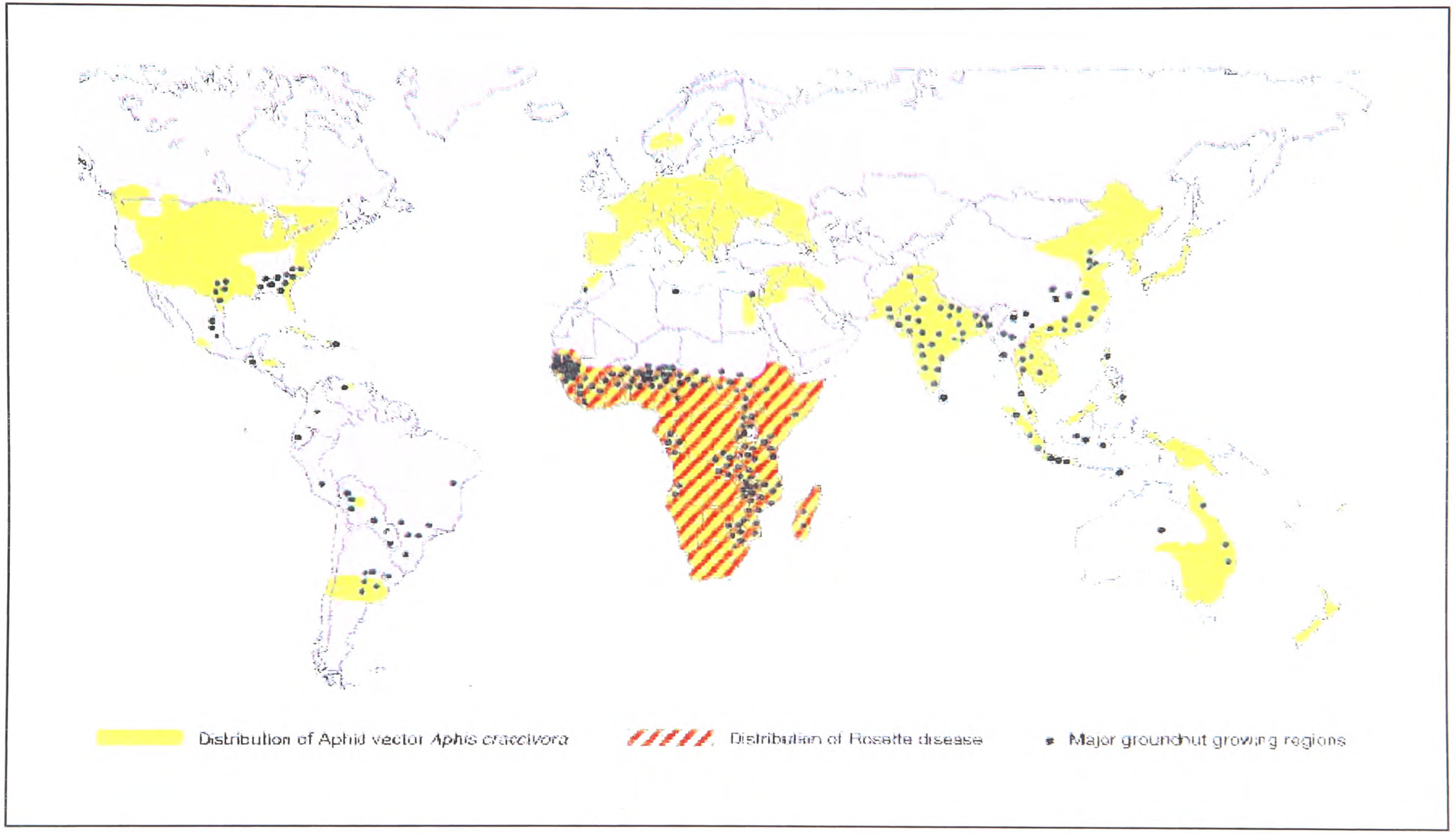


Figure 1 9: World-wide distribution of groundnut cultivation, groundnut rosette disease and the aphid vector, *Aphis craccivora*.

1.2.4.3. Disease-vector relationship

Most of our current knowledge about the persistent circulative transmission of luteoviruses (Fam. *Luteoviridae*) and vector specificity resulted from the work of W.F. Rochow and F.E. Gildow (Rochow, 1970; Gildow, 1987; 1990). Their model system involved cereal aphids (*Rhopalosiphum padi*, L.) and the transmission of *Barley yellow dwarf virus* (BYDV). All luteoviruses infect and replicate only in the sieve elements and parenchyma or companion cells of the phloem located in the vascular bundles of host plants (Waterhouse *et al.*, 1987).

Characteristics of a persistent circulative transmission mode include:

1. no virus replication in the vector;
2. a circulative movement of the virus particles within the vector and final storage in the accessory salivary glands;
3. the ability to transmit the virus for up to 14 days, and possibly for life, in all stages of the vector (Gildow, 1987).

Groundnut rosette is of particular interest because GRV and satRNA must be packaged within the coat protein of the luteovirus, which is GRAV to be aphid-transmissible. This means that all virus particles must be acquired from the phloem into the aphid's body via the food canal (Figure 1.10). The aphid foregut is chitin-lined, preventing the possibility of virus penetration through this tissue and the virus particles continue to pass through the anterior midgut and posterior midgut and into the hindgut. Much of the virus probably continues to flow out of the aphid in the honeydew and is then unavailable for acquisition or transmission (Gildow, 1990). The hindgut is a very thin-walled region of the alimentary canal and virions moving through the hindgut come into contact with the apical plasmalemma and attach to the membrane. Virus uptake is initiated by cellular endocytosis, packaged in transport vesicles and moved through the cell cytoplasm to the other side of the gut and released into the aphid's haemocoel.

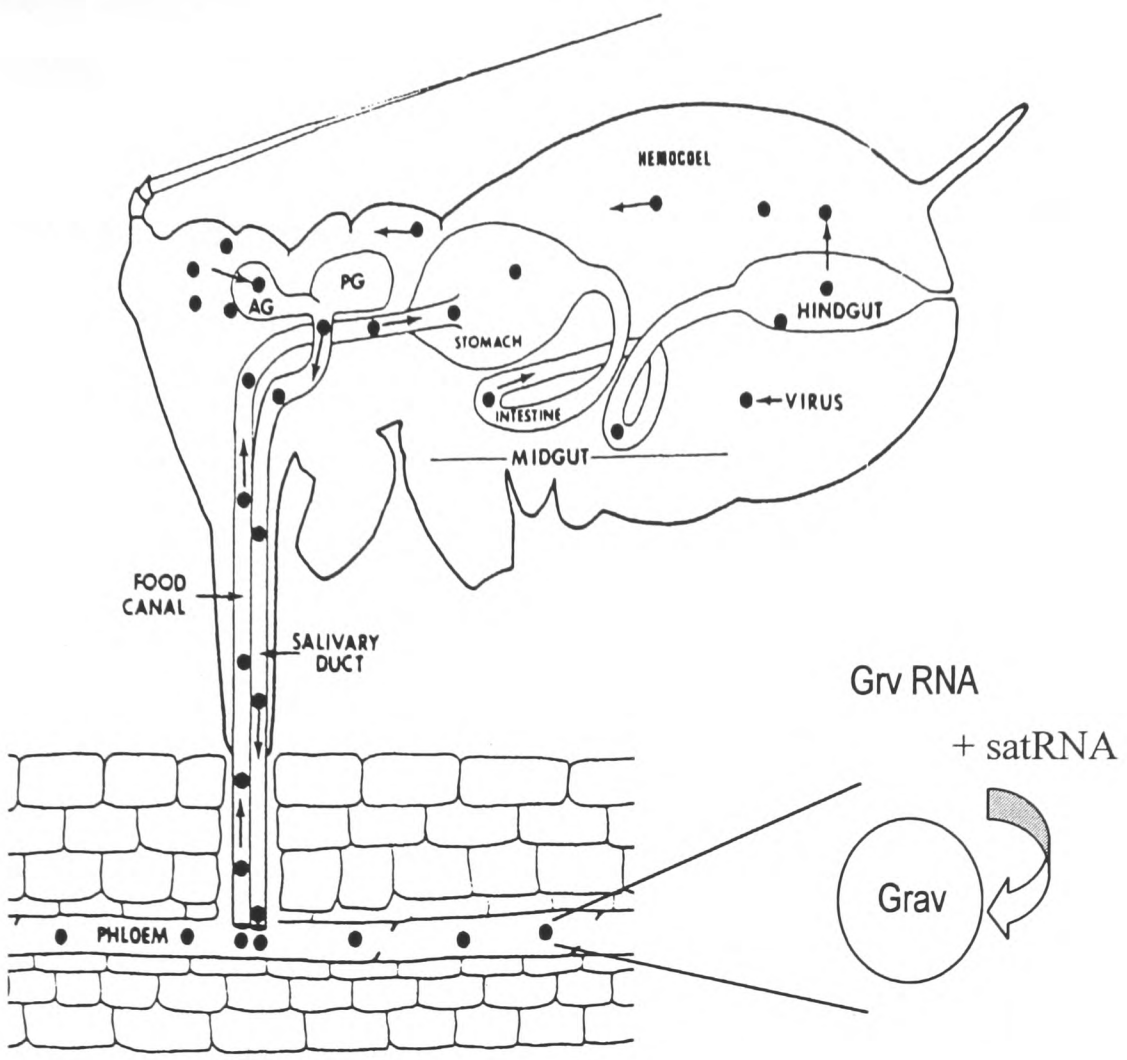


Figure 1 10: Aphid feeding and acquisition of viral particles in the plant tissue (Gildow, 1987). Virus particles can only be acquired when the insect is feeding on the phloem. GRV and its satRNA can only be acquired when encapsidated into the coat protein of GRAV. The particles are transported through the midgut to the hindgut where they diffuse into the haemocoel. From there they move to the front and are stored in the accessory salivary gland. This is possible through specific interactions between the coat protein and the membrane of the accessory salivary gland.

Virus acquisition of all three GRD virus agents does not necessarily result in their transmission (Naidu *et al.*, 1999a). Separate infections with GRAV or with GRV + satRNA following aphid transmissions from plants infected with all three agents have been consistently observed in the laboratory and field (Murant, 1990; Naidu *et al.*, 1998, 1999a) (Figure 1.11). While GRAV must be inoculated into the phloem or parenchyma cells, GRV and satRNA infection can also occur during exploratory probes into the cells of the epidermis and mesophyll (Naidu *et al.*, 1999b). The infection with luteoviruses

increases with the number of viruliferous aphids used (Naidu *et al.*, 1999a) and has also been observed for other circulative transmitted viruses including BYDV (Gildow., 1990).

1.2.4.4. Diagnosis

In the field groundnut rosette disease can be diagnosed based on symptom expression in the plants. Two forms can be distinguished as green rosette and chlorotic rosette (Smartt, 1961). Chlorotic rosette is ubiquitous in SSA, while the distribution of green rosette is patchy possibly because the symptoms are less apparent. Symptoms of either form include severe stunting due to shortened internodes and reduced leaf size, leading to a bushy appearance. However, in some West African countries, symptoms of green rosette resemble symptoms of peanut clump disease (Naidu *et al.*, 1997) making it difficult to differentiate and determine the distribution and impact of groundnut rosette disease on groundnut production. To confirm the presence of the disease, it is therefore important to test for the three agents of GRD using diagnostic tests. Improved methods include Triple Antibody Sandwich Enzyme Linked Immunosorbent assay (TAS-ELISA) for GRAV detection (Rajeshwari *et al.*, 1987), a Dot Blot Hybridisation (DBH) assay for detection of GRV and satRNA (Blok *et al.*, 1995) and Reverse Transcription Polymerase Chain Reaction (RT-PCR) that allows detection of each of the three agents (Naidu *et al.*, 1998). More details about RT-PCR and detection of groundnut rosette disease agents are described in Chapter 4.

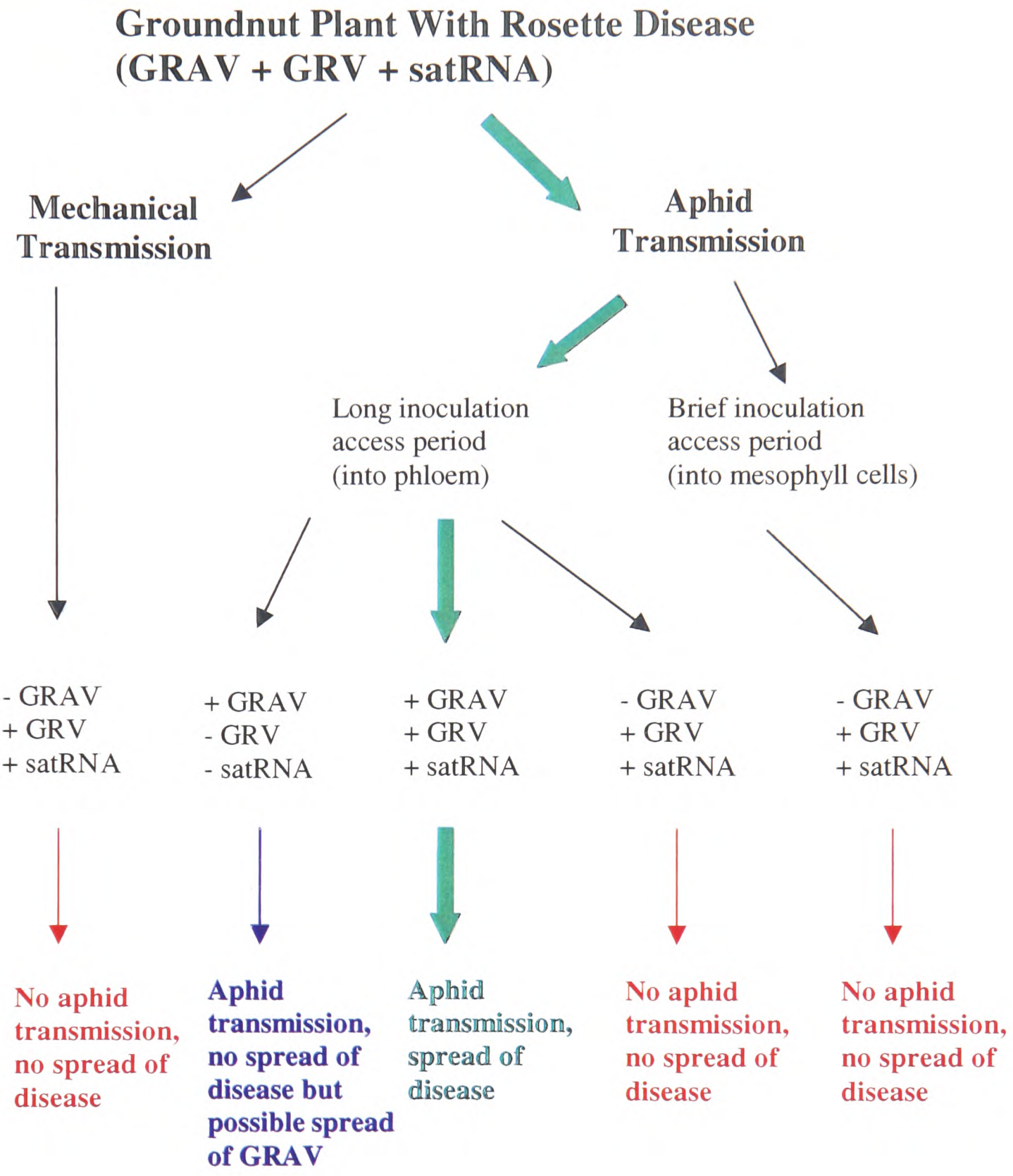


Figure 1 11: Separation of groundnut rosette disease in time and space during mechanical or aphid inoculations. Aphids fail to transmit the disease in absence of *Groundnut rosette assistor virus* (GRAV) and plants lacking *Groundnut rosette umbravirus* (GRV) and satellite RNA (satRNA) do not show disease symptoms. + indicates replication, - indicates no replication and green arrows indicate epidemiological significance (Figure is modified from Naidu *et al.*, 1999b).

1.2.4.5. Management of GRD

The implementation of cultural methods and seed-based technologies may not be effective for all systems but for the management of GRD, it proved to be highly effective.

Viricides

So far no viricides have been marketed because of their ineffectiveness, phytotoxicity and costs (Wightman *et al.*, 1990). Therefore, the majority of control measures for viruses are indirect, aimed at reducing sources of inoculum within and outside the crop, to limit spread by vectors, and to minimise the effects of infection on yield.

Insecticides

Organophosphate pesticides have been used to control aphid populations and the subsequent spread of GRD in the field (Davies, 1975a,b; Evans, 1954; Soyer, 1939). The timing, dosage and type of insecticide applications are critical for effectively diminishing the vector population and require an early forecast of vector migration into the crop (Naidu *et al.*, 1998). Use of insecticides as a control measure is usually not economically feasible for smallholder farmers in SSA and, if available, includes potential hazards as a result of inappropriate use of the chemicals. In addition, the delicate balance between aphid vector and natural enemies might change and the use of chemicals can result in the development of resistant biotypes. Considering the toxicity and potentially detrimental effect of insecticides on the environment, other low-input integrated methods of aphid and disease management such as improved cultural practices and resistant varieties are preferable.

Cultural methods

Early sowing and maintaining uniform dense stands of groundnut greatly reduce the incidence of rosette disease (Booker, 1963; A'Brook, 1964; Farrell, 1976a,b; Davies, 1976). Early-sown crops largely escape infection and the greater virus incidence in later-sown crops is probably a function of the timing of vector flights (A'Brook, 1968) and preference for the young crop. Management by early planting and dense spacing continues to be satisfactory in the few parts of SSA where large-scale commercial

farming is practised. However, widespread adoption among smallholder farmers is seldom feasible because of the preferred early sowing (many farmers sow cereal crops first), labour constraints and costs, and/or insufficient seed to allow even moderately dense populations. Furthermore, early planting, in many areas, necessitates harvesting the crop during wet weather, causing problems of drying and predisposition to moulds (Naidu *et al.*, 1999b).

Genetic resistance

The rapid development of resistance to pesticides in insects, the high research and development costs of new chemicals and subsequent testing and the public awareness of the hazards of pesticide use, all contributed to creating interest in breeding for vector resistance in plants (Gibson & Plumb, 1977; Dunn, 1978; Jones, 1987). Planting resistant material is one of the most effective, economical and environmentally safe management tactics. By definition host plant resistance is

> "*...the relative amount of heritable qualities possessed by the plant which influence the ultimate degree of damage done by the insect in the field...*" (Painter, 1951).

In 1990, EC36892 (ICG5240) was reported to be the most consistently vector resistant groundnut variety in Southern India and Malawi and the most promising source of vector resistance for further breeding programmes (Padgham *et al.*, 1990a). The resistance to the vector demonstrated to be a protection against GRV +satRNA infection even under abnormally heavy infestations by viruliferous aphids (Padgham *et al.*, 1990b). However, breeders at this time did not originally accept the variety in their improvement programmes because of its perceived unsatisfactory agronomic traits, such as low yield and growth characteristics (F.M. Kimmins pers. comm.).

1.2.4.6. Gaps in knowledge

Although *A. craccivora* has been studied extensively since 1960, information is lacking on the various topics that could be important in the development of disease management strategies for groundnut rosette.

Primary infection

The source of viruliferous aphids that initiate groundnut rosette disease (primary infection) is unknown. Groundnut rosette is considered a polycyclic disease because each infected plant serves as a source for initiating subsequent spread in the field (Thresh, 1983a). Since the viral agents are not seed-borne, the primary infection must be introduced into the crop by viruliferous aphids. In areas with two growing seasons such as Uganda, the disease complex could be sustained on volunteer plants. Volunteer plants are groundnut plants germinating from pods that remained in the soil after harvest of the first season. They can harbour the viral agents and aphid vector from where they can initiate sources of primary infection. In areas with only one growing season -such as Malawi (December-May)- the disease might be maintained on groundkeepers during the dry season (June-November). During this period these plants remain very small and could support the disease agents and serve as source plants for virus transmission in the next season. It has also been suggested that aphids survive on a succession of dry season hosts, particularly shrub and tree species that are common in the groundnut growing regions of Africa and that produce flushes of new growth before the onset of the rains (Adams & Farrell, 1967). These host plants could support aphid colonisation but must also be reservoir hosts for the rosette disease agents. In 1966, Okusanya and co-workers showed that virus could be acquired by the aphid when given access on *Trifolium repens* suggesting that leguminous weeds may act as reservoirs. This work has not been repeated. It has also been postulated that plant species that are not preferred by *A. craccivora* may still become infected during exploratory probes, provided that they are hosts for the viral components of the rosette disease complex. Such plants may support replication only of GRV and its satRNA and would not be sources for further transmission through the absence of GRAV. Alternatively, all three components may replicate, but the plants may be poor sources for subsequent spread because of the need for extended virus acquisition feeding periods.

Alternatively, it was suggested that viruliferous immigrants arrive from neighbouring countries that receive rains earlier and are carried on the prevailing winds (R.A. Cheke pers. comm., see also atmospheric movement).

Secondary spread

Once the primary infection is established, infected plants form a source for aphids to acquire the virus and subsequent transmission of the disease (Figure 1.12). The development of winged morphs and the high mobility of adults and nymphs can cause a rapid spread of the disease to neighbouring plants and fields (secondary spread). The nature and pattern of the disease spread can be influenced by plant age, crop density, timing and efficiency of transmission by viruliferous aphid vectors that reach the crop, proximity to the source of primary inoculum, climatic factors and predators and parasitoids of vector populations within the crop (Naidu *et al.*, 1999b). Detailed studies of the population dynamics, the role of plant genotype, natural enemies and the stages at which individual movements to neighbouring plants occur, could be helpful in disease management strategies. It has also been suggested that the symptoms can attract new immigrants and that the aphids' fecundity is higher on diseased plants resulting in fast population build up and subsequent dispersal of viruliferous aphids (Réal, 1955). However, it is only the plants containing all three virus agents that play a crucial role in the secondary spread of the disease, while the plants showing disease symptoms, irrespective of the presence of GRAV, influence yield losses.

Plants infected with groundnut rosette virus disease in the field in Malawi

Figure 1 12: Secondary spread of groundnut rosette disease by aphids moving throughout the crop from a primary infection site (from Naidu *et al.*, 1999b).

Atmospheric movement

Another unresolved issue responsible for the large and unpredictable fluctuations in the disease incidence is the wind-borne dispersal of aphids. Once small insects have left the surface boundary layer long distance migration occurs over a range of tens or even hundreds of kilometres. Compared to vector movements in Europe, North America and Eastern Asia, very little is known about the atmospheric transport of aphids in Africa. High-altitude trappings in Africa (Rainey, in Thresh, 1983b) suggested that *A. craccivora* has the potential to disperse over long distances and it was suggested that alatae of *A. craccivora*, originating from areas that receive rains and are planted earlier, are carried on prevailing winds and deposited along a zone of wind convergence in areas where the rains and planting have just started. Such depositions have been described for the desert locust *Schistocerca gregaria* (Forsk) and African armyworm *Spodoptera exempta* (Wlk.) (Betts, 1975) also using new technologies such as Geographic Information Systems (GIS) and radar-based technologies (Day *et al.*, 1996), but it is not clear whether it is also true for *A. craccivora* (Cheke, 2000).

Existence of biotypes

There is a need to better understand the existence of biotypes of *A. craccivora* as the incidence of biotypes complicates the management and control of the aphid and hence the disease it transmits. Despite the broad host range, different host plant preferences seem to exist and aphids which were reared on one host, such as cowpea, do not seem to rapidly colonise a second host, e.g. groundnut (Chapter 3). Genetic markers for the biotypes of *A. craccivora* could be used to monitor their abundance and distribution. It is important to determine the transmission efficiencies as different biotypes can have different abilities to acquire and transmit viruses (Saxena *et al.*, 1964). This information can be used in the development of management programmes to minimise the incidence and spread of particular aphid biotypes. However, the biotype concept is a controversial topic and is described in more detail in Chapter 7.

1.3. Host plant selection by aphids

As this thesis is focused on studying the value of vector resistance in improved varieties of groundnut, it is important to discuss aphid host plant selection behaviour. Generally, host plant selection is a chain process that starts with orientation activities to find a plant from a distance and ends with plant acceptance, feeding and reproduction or rejection. The selection process involves responses to visual and olfactory cues, alighting on the plant, exploring the leaf surface, probing and penetration of plant tissues by the stylets to locate the phloem sieve elements and testing the phloem contents. At each stage of the process a balance between positive and negative stimuli may influence the sequence and ultimately the insect may leave or stay (Figure 1.13). Plant species recognition and plant quality assessment form the basis of the dual discrimination theory of host selection (Kennedy & Booth, 1951).

1.3.1. Aphid response to colour

Moericke (1955) recorded colour as a major distance factor for aphid attraction to plants. Aphids respond to different wavelengths of light differently depending upon their development stage, the degree of population density, the suitability of their plant and other environmental conditions such as temperature and wind speed (Klingauf, 1989; Robert, 1989). Winged adult aphids commonly enter a phase in which they reject their old host and become attracted to the shorter blue-ultraviolet light of the sky. When the conditions are favourable, the aphids will takeoff and enter a migratory phase until they enter the cruising phase where horizontal flight becomes more frequent (Robert, 1989). Throughout this phase, longer wavelengths of light become more attractive and they will orientate themselves toward the orange-yellow-green light reflected by plants (Moericke, 1962; Müller, 1964; Gibson & Rice, 1989; Robert, 1989). These colour cues will also be combined with other visual cues such as the size, shape and contrast of plants or other objects against their background. Aphids prefer large objects or plants over smaller ones and plants or objects that contrast with their surroundings, rather than blend in with them, and crops that are sparsely spaced rather than dense (Gibson & Rice, 1989; Dunn, 1969).

1.3.2. Aphid response to plant odours

It was long accepted that aphids do not make use of chemical cues in their choice of a host plant after flight. Host plant selection took place after a visually directed non-specific landing (Kennedy *et al.*, 1959a,b). Although responses to sex and alarm pheromones have been demonstrated (Pickett *et al.*, 1992) there are only a few reports on odour attraction (Chapman *et al.*, 1981) or repellence in the field (Cambell *et al.*, 1993; Pettersson, 1993). The discussion on the role of host-plant odours has been renewed on laboratory studies on aphid olfactory receptors and olfactometer experiments (Pickett *et al.*, 1992; Hardie *et al.*, 1994; Pettersson *et al.*, 1995; Visser & Piron, 1995)

1.3.3. Leaf surface exploration

Once an aphid has landed on the plant, it explores its surface. Chemicals of the plant surface (e.g. components of the epicuticular layer, trichomes) are perceived by antennal chemoreceptors (Bromley *et al.,* 1979) and by receptors on the tibiae and tarsi (Anderson & Bromley, 1987). This behaviour is associated with the testing of the chemical nature of the surface and outer tissues of the plant. Chemicals found within plant cuticular waxes are thought to have a direct involvement in host selection and, in many cases, are insect-host specific (Klingauf *et al.*, 1978). Physical and chemical interference by trichomes on insect movement and feeding can be effective (Gunasinghe *et al.*, 1988; Tingey *et al.*, 1981). As aphids lack external contact chemoreceptors (Tjallingii, 1978b), internal plant factors encountered during stylet penetration in epidermal, mesophyll and phloem tissues are generally considered to be the main cues used by aphids to accept or reject a plant (Pollard, 1973; Montllor, 1991; Harrewijn, 1990).

1.3.4. Stylet insertion for phloem sieve element location

When probing, the plant's internal fluids are tasted by the gustatory papillae of the epipharyngeal organ (Wensler & Filshie, 1969), which is positioned on the dorsal side of the pharyngeal duct within the aphid's head at the anterior end of the food canal

(Ponsen, 1989). Stylet pathways are predominantly intercellular; although many brief intracellular punctures followed by stylet withdrawal can often be seen (Tjallingii & Hogen Esch, 1993). Before reaching the phloem, aphids and other phloem feeding insects encounter secondary metabolites (allelochemicals) during these intracellular punctures, presumably from the cytoplasm and vacuole (Martin *et al.*, 1997). A wide variety of such compounds are known mediators of aphid behaviour and act as deterrents or phagostimulants (Schoonhoven & Derksen-Koppers, 1976) or cause antibiotic effects (Pollard, 1973; Montllor, 1991). Although there are no experimental data reported on sampling of intercellular fluids, it is widely assumed that it does occur and that the fluids are tasted by the epipharyngeal gustatory organ (Tjallingii, 1978b).

1.3.5. Testing the phloem content

The major food source of the Aphididae is commonly known as 'sieve tube sap' (Miles, 1999). They are known to feed passively on the sap that is driven into the mouth-parts by the turgor pressure in the sieve tubes (Emden van, 1988). When the stylets reach a sieve element, salivary enzymes are injected counteracting sieve element reactions such as phloem protein gelation, callose deposition around the stylet, which have been suggested to function as fast wound reactions and to seal individual sieve elements (Dixon, 1975; Walsh & Melaragno, 1981; Evert, 1990; Tjallingii & Hogen Esch, 1993). The phloem content generally consists of free amino acids and high concentrations of sugars (commonly sucrose). Secondary substances may be important as additional feeding stimulants or deterrents (Klingauf, 1989) and the final acceptance of a plant as a host depends on the qualitative and quantitative properties of the phloem sap.

Different stages of the host plant selection process of *A. craccivora* on groundnut varieties of interest are further examined in the chapters as indicated in figure 1.13. The focus lies on variety ICG12991, which has been described as resistant to the aphid vector of groundnut rosette disease (Merwe van der *et al.*, 2001).

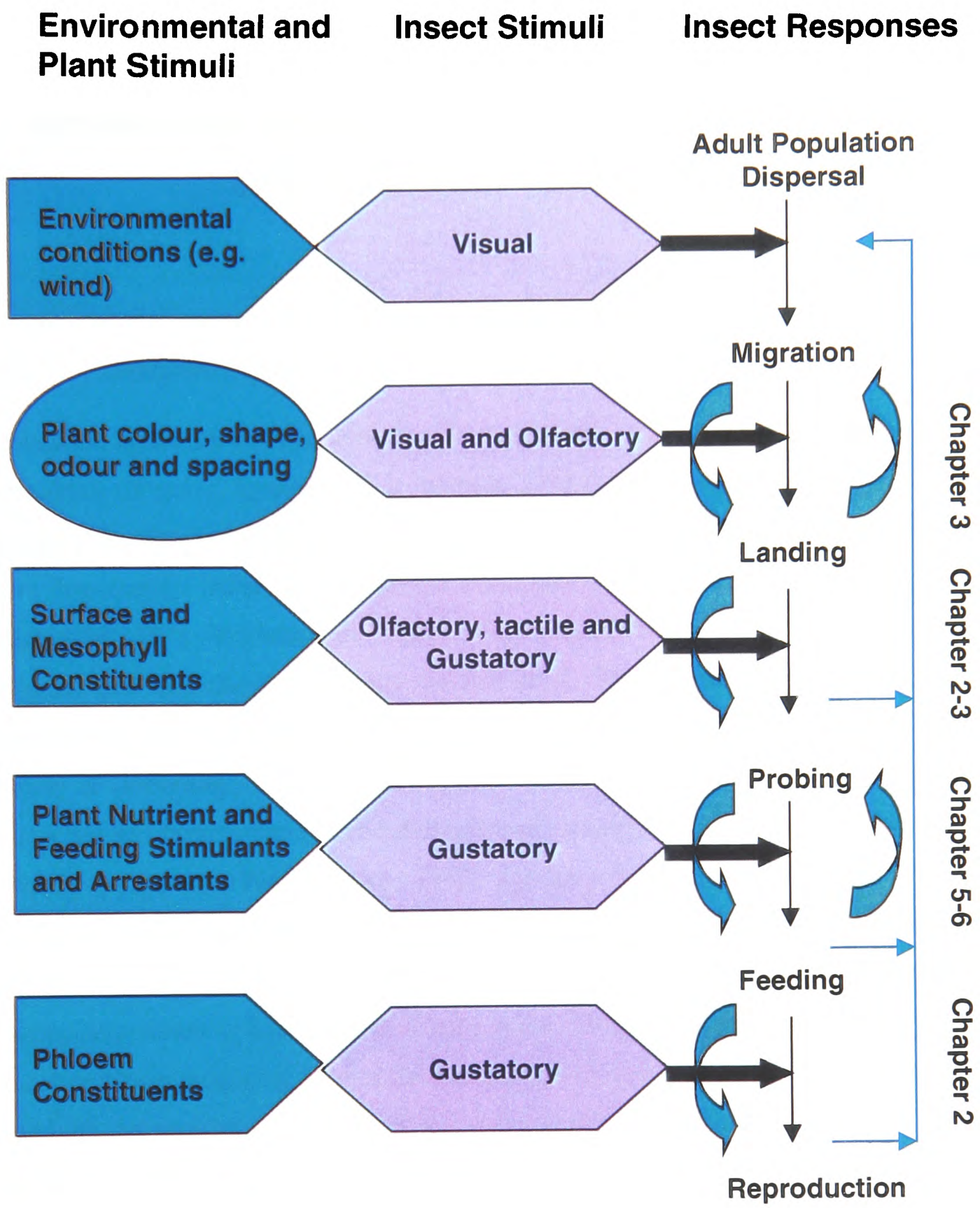


Figure 1 13: A generalised model for the host plant selection process based on environmental, plant and insect stimuli in phytophagous aphids. Thin black arrows indicate the sequence of the selection process. The green arrows indicate the choice aphids can make between the different phases.

CHAPTER 2

APHID PERFORMANCE ON GROUNDNUT UNDER LABORATORY CONDITIONS

2.1. Introduction

Groundnut aphid or cowpea aphid, *Aphis craccivora* Koch, is an economic pest of groundnut (*Arachis hypogaea* L.), cowpea (*Vigna* unguiculata, Walp.) and other legumes. It causes yield losses either directly by feeding and draining phloem sap but, more importantly, indirectly through the transmission of several viruses (Feakin, 1973; Singh *et al.*, 1978; Wightman & Amin, 1988). It is the vector of at least seven viruses of groundnut including *Groundnut rosette virus*, which continues to be the most important biotic constraint for groundnut production in sub-Saharan Africa (SSA) (Storey & Bottomley, 1928). Reducing populations of the vector through insecticide-sprays has had limited success as a management strategy for the disease in small-holder systems (A'Brook, 1964; Booker, 1963; Davies, 1975a,b, 1976; Farrell, 1976a,b; Guillemin, 1952; Subrahmanyam & Hildebrand, 1994), because it is not economically feasible for small-holder farmers and improper use could lead to the development of insecticide-resistant aphid biotypes (Naidu *et al.*, 1999b). Host plant resistance to *A. craccivora* in groundnut is recognised as a potentially effective and economic method to limit both the population build-ups of the vector and the virus it transmits (P.J.A. van der Merwe pers. comm.).

Several definitions of host plant resistance to insects have been put forward (Beck, 1965; Harris, 1979; Ponti de, 1983) and the most widely accepted is that by Painter (1951); given on page 23. In agriculture, it represents the ability of a certain variety to produce higher yields of good quality than other varieties under the same level of insect infestation and in a comparable environment. Generally, three types of resistance to insects are accepted which are defined as follows:

1. Antixenosis is the resistance mechanism employed by the plant to direct insects away from the host plant. It is not strictly the same as non-preference, which was originally defined by Painter because that term describes an insect behavioural response rather than a plant mechanism (Kogan & Ortman, 1978).
2. Antibiosis is the resistance mechanism that operates after the insects have colonised and have started utilizing the plants. Delayed insect development, decreased size, impaired or lowered reproduction and reduced survival are responses most often observed (Painter, 1951).
3. Tolerance is a genetic trait of a plant that protects it against an insect population which would damage a susceptible host variety (Painter, 1951).

It is not always clear which mechanism is active against the insect and a range of observations on insect behaviour in choice and no-choice experiments is required. Also, laboratory-based observations must be compared with field studies because the expression and magnitude of resistance can be affected by environmental conditions.

In this study, methods to investigate aphid behaviour and performance on groundnut plants were designed and tested on a range of selected groundnut varieties, which were of interest in future breeding programmes (Chiyembekeza *et al.*, 1997; Naidu *et al.*, 1999b). The intrinsic rate of natural increase (Rm) and the survival of *A. craccivora* on a range of varieties were assessed in no-choice experiments, while plant colonisation using one resistant and one susceptible variety was investigated in choice-experiments.

2.2. Materials and Methods

2.2.1. Growing and rearing conditions for aphids and plants under controlled laboratory conditions

2.2.1.1. Plants

The plants were grown separately from the insects at the Natural Resources Institute, University of Greenwich, under the following conditions:

Light (L)/Dark (D): 12h/12h

T: 29°C ± 1°C (L) / 25°C ± 1°C (D)

Relative Humidity (RH): 50% ± 5% (L) / 60% ± 5% (D)

The groundnut seeds were planted in plastic pots by pressing the seeds ino the soil (John Innes No.2) and cover them by a small layer of soil (0.5cm). The age of plants was then based on the days after planting (DAP), which is from the day that the seed is planted in the soil. Seedlings emerged after 6 days.

Six groundnut varieties were selected to investigate aphid performance (Table 2.1). ICG12988 was discarded for further study due to uncertainty regarding its pedigree (P.J.A. van der Merwe pers. comm.). ICG12991 and JL24 were directly compared in some experiments because both are Spanish types, characterised by an erect growth habit, sequential branching pattern and short maturation period (90-110 days). In contrast, Virginia types such as CG7 and ICG SM90704 have a more spreading growth habit, alternate branching pattern and medium to late maturation period (120-180 days).

2.2.1.2. Insects

Insects were reared under the same conditions as the plants (2.2.1.1). Separate cylindrical perspex cages were used to rear colonies of aphids originating from Malawi and Uganda. Alate aphids (winged morphs) were used to start fresh colonies, as they tend to give birth to apterous aphids (non-winged morphs) (Lees, 1966; Pers. observ.).

Newly moulted adult aphids (<24h) were then used for the experiments. As no differences were observed between the two aphid clones, some experiments were restricted to the use of the Ugandan aphid colonies.

Table 2 1: Groundnut varieties selected for evaluating aphid performance in the laboratory with their classification as botanical variety.

Variety	Botanical variety	Aphid susceptible/resistant
CG7	Virginia	Susceptible
ICG-SM90704	Virginia	Susceptible
ICG5240	Virginia	Moderately resistant*
JL24	Spanish	Susceptible
ICG12991	Spanish	Unknown
ICG12988	Spanish	Unknown

*: Padgham *et al.*, 1990b

2.2.2. No-choice experiments to evaluate parameters for aphid population growth and fitness

The following experiments were carried out in a constant temperature and RH room in the same conditions as for rearing insect colonies (2.2.1).

2.2.2.1. Intrinsic rate of increase of *A. craccivora* on groundnut

The intrinsic rate of increase (Rm) is a measure of the natural rate of increase of an insect population (Birch, 1948). Wyatt & White (1977) simplified the method and proposed an equation (1) to estimate the population increase rate specifically for aphids.

$$Rm = 0.74 \text{ X } (\text{Log}_e \text{ Md}) / D \qquad (1)$$

where D = Nymphal development time in days

and Md = Number of offspring produced in time D

To obtain data on D and Md, the following experimental design was developed. The top of individual plants containing the youngest stages were enclosed in a perforated crisp bag (Cryovac Europe, St.Neots, UK), which while insect-proof, did not restrict photosynthesis and protected the plants from other greenhouse insects such as thrips. Plants of 7 days after planting (DAP) were totally enclosed in the crisp bags. The crisp bags were sealed with paperclips in order to manipulate the plants and aphids easily. On day 0, one adult apterous aphid per plant per variety was placed on the youngest leaf using a fine camel-hair brush. The next morning on day 1, the adult and all but one first instar nymph were carefully removed. The isolated nymphs were monitored daily to determine the time taken to reach the reproductive stage (D). Then, the fecundity of each individual aphid was recorded during a period equal to D to determine Md. After 4 days, late instars were removed to prevent them reaching adulthood and becoming confused with the experimental reproductive adults. The Rm was calculated for aphids on 6 groundnut varieties of two plant ages (7 and 28 DAP). The varieties were CG7, ICG SM90704, JL24, ICG5240 (also known as EC36892), ICG12988 and ICG12991

A randomised block design with 5 plants per variety was used and repeated 4 times. Both plant ages and aphid clones were tested independently of each other. Data on D and Md were SQRT transformed prior to the statistical analysis (ANOVA with contrasts in GENSTAT 4.1, 6th edition) but the outcome did not differ from non-transformed data. Values of Rm were not transformed.

2.2.2.2. Survival of aphid nymphs on groundnut

A similar experimental design as described in 2.2.2.1. was applied to investigate aphid survival on a range of groundnut varieties at 7 DAP. All first instar nymphs produced on day 1 by one adult apterous aphid per plant per variety were allowed to develop on the enclosed part of each plant. The number of first instar nymphs was recorded at day 1 and the percentage of surviving nymphs was calculated at day 5. The experiment was repeated on plants at 28 DAP. A randomised block design containing 5 plants per variety was repeated 4 times. The percentage of surviving insects was analysed with Logit Analysis in GENSTAT 4.1 (6th edition).

2.2.2.3. Weight of adult aphids that had developed on groundnut

The weights of adult apterous aphids, which had developed on 4 different groundnut varieties including CG7, ICG SM90704, JL24 and ICG12991 (10 DAP) were measured on a Mettler AT201 balance (sensitivity of 0.01mg). One plant per variety was considered to represent a block and 5 blocks were placed in a randomised design. Five first instar nymphs per plant were left to develop to adulthood (=25 aphids per variety) and then placed in a freezer at -80°C. The frozen aphids were weighed while being prevented from thawing. Weight measurements were subjected to Analysis of Variance.

2.2.3. Choice experiments

2.2.3.1. Aphid distribution on plants of two varieties

One adult apterous aphid was placed on the highest part of the plant (14 DAP) and the position of the aphid was recorded at intervals of 6 and 24 hours. After 24 hours the adults were removed. The positions of the nymphs were then recorded and again at 96 hours. Two varieties, JL24 and ICG12991, were selected for this experiment and observations were only made for a few intervals in order to prevent displacement of the aphids while handling. The experiment was repeated 12 times. The plant parts were divided as follows (Figure 2.1).

P: Petiole: stalk of every leaflet that attaches to the main stem or side branches of the main stem

S: Main stem

SB: Stem-branches between the leaflets

B: Bud of developing leaflets

L: Older leaf tissue

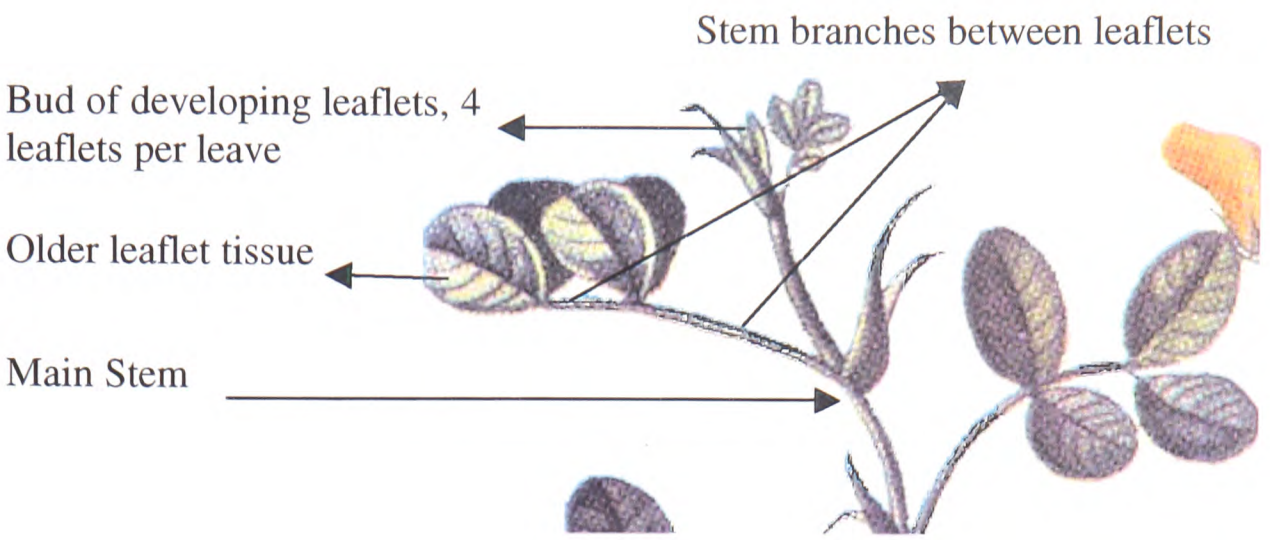


Figure 2 1: Plant parts of groundnut as used in aphid choice experiment
www.vodou.org/images/herbs/ arachide_hypogee.jpg

2.2.3.2. Aphid distribution between plants of two varieties

Plastic pots containing test plants of ICG12991 and JL24 were randomly placed in the four corners of a transparent container (arena) (27.5 x 15.5 cm). Each arena contained two plants of each variety at a similar growth stage. Three different age groups of the plants (10 DAP, 17 DAP and 28 DAP) were tested independently of each other and replicated 10-14 times. The container was filled with soil to cover the pots and the inner rim of the container was treated with polytetrafluoroethylene (Fluon, Whitfor plastic Ltd) to prevent the aphids escaping from the arena. As an additional precaution, sticky tape was placed around the edges of the arena to catch escaping insects. A Petri dish (4 cm diameter) containing 20 apterous aphids, which had been deprived of food for 3 hours to stimulate their searching behaviour, was placed into the soil with the edges at soil level in the centre of the arena. The distribution of the aphids on the plants of JL24 and ICG12991 was recorded at regular intervals over a 24h period. Aphids which were moving in between the plants and those which were captured on the sticky tape were defined as 'rest'. The number of nymphs produced on the different varieties was also counted at the end of the experimental period. The experiments were conducted in a light and temperature-controlled room.

Data were arcsin transformed prior to ANOVA because the proportion of aphids on each of the varieties could be calculated. The numbers of nymphs produced on both varieties were compared using the Mann-Whitney *U* test.

2.2.4. Additional screening for vector resistance on varieties originating from India and China.

A range of varieties collected from India and China (Virginia type) was reported as groundnut rosette disease resistant (Subrahmanyam *et al.*, 1998; Table 2.2). This disease does not occur in these regions and therefore they were screened for vector resistance in the laboratory at NRI-UoG after a suitable technique was developed (2.2.2.1). The Rm of aphids was evaluated on the varieties originating from India (ICG9723, ICG11735, ICG11788) and from China (ICG11649). Control varieties were the susceptible varieties JL24, CG7 and ICG SM90704 and the resistant varieties ICG12991 and ICG SM99540. ICG SM99540 was selected after fieldwork in Uganda indicated a strong level of resistance to the aphids (Chapter 3).

Plants were 21 DAP and divided in 3 blocks of 5 plants per variety. Aphids originated from the Ugandan clone. Data on nymphal development time, adult fecundity and rate of population increase were analysed with ANOVA and contrasts in GENSTAT 4.1 (6th edition).

Table 2 2: Groundnut rosette disease incidence on groundnut varieties originating from India and China in field screening trials during the 1995/1996 and 1996/1997 growing seasons at Chitedze, Malawi (modified from Subrahmanyam *et al.*, 1998).

Variety	Country of origin	Rosette Disease Incidence (%)		
		1995/1996	1996/1997	Mean
ICG9723	India	15	9	12
ICG11735	India	7	7	7
ICG11788	India	3	2	2.5
ICG11649	China	7	2	4.5
ICG SM99540	India	*	*	*
ICG12991	India	0	6	3
JL24	India	93	92	96.3

* : no data.

2.3. Results

2.3.1. No-choice experiments to evaluate parameters for aphid population growth and fitness

2.3.1.1. Intrinsic rate of increase of *A. craccivora* on groundnut

Tables 2.3a,b show the intrinsic rate of natural increase (Rm) of aphids from the Ugandan clone and the Malawian clone, calculated according to equation 1 (2.2.2.1). The mean nymphal development times and fecundities are presented in Table 2.4a,b. The Rm of both aphid clones was significantly lower on the varieties ICG5240, ICG12991 and ICG12988 ($P<0.01$; ANOVA with contrasts), irrespective of plant age. The lower Rm values were attributable to both an increased nymphal development time (D) and reduced fecundity (Md) on ICG12991 and ICG12988 ($P<0.001$). On ICG5240, reduced fecundity was the main factor responsible for the lower Rm values ($P<0.01$). Rm values could not be calculated for all aphids, mainly due to mortality, as will be further outlined in 2.3.1.2. This explained the differences in number of replicates for each variety, which were the lowest for ICG12991 and ICG12988.

The mean D for both aphid colonies on 7 DAP plants of varieties CG7, JL24 and ICG SM90704 and ICG5240 (7 DAP) was approximately 5 days whereas on ICG12991 and ICG12988 the mean D was 5.5 days. On older plants (28 DAP), D was 6 days on CG7, JL24 and ICG-SM90704 and 7 days on ICG5240, ICG12991 and ICG12988.
The mean Md for aphids on 7 DAP plants of varieties CG7, JL24 and ICG-SM90704 was approximately 70 compared to 55 on ICG5240 and 40 on ICG12991 and ICG12988. The Md was lower when plants were older (28 DAP) for all 6 varieties and was approximately 60 for aphids feeding on the former three varieties, 40 on ICG5240 and 25 on ICG12991 and ICG12988.

Table 2 3: The intrinsic rates of increase (Rm) of *A. craccivora* on 6 groundnut varieties of two different ages (7 DAP and 28 DAP). Aphids were collected from a Ugandan (a) and from a Malawian population (b).

a)

Ugandan aphid population

	Plants 7 DAP		Plants 28 DAP	
Variety	**Rm ± S.E.**	**n**	**Rm ± S.E.**	**n**
CG7	0.62 ± 0.01^{a}	20	0.51 ± 0.01^{a}	17
ICG SM90704	0.62 ± 0.00^{a}	20	0.53 ± 0.01^{a}	20
JL24	0.62 ± 0.01^{a}	20	0.50 ± 0.01^{a}	17
ICG5240	0.56 ± 0.01^{b}	20	0.39 ± 0.02^{b}	15
ICG12988	0.45 ± 0.01^{c}	20	0.32 ± 0.02^{c}	9
ICG12991	0.44 ± 0.01^{c}	20	0.34 ± 0.02^{c}	8

b)

Malawian aphid population

	Plants 7 DAP		Plants 28 DAP	
Variety	**Rm ± S.E.**	**n**	**Rm ± S.E.**	**n**
CG7	0.62 ± 0.01^{a}	20	0.52 ± 0.012^{a}	17
ICG SM90704	0.62 ± 0.01^{a}	20	0.52 ± 0.01^{a}	15
JL24	0.61 ± 0.01^{a}	20	0.53 ± 0.01^{a}	18
ICG5240	0.57 ± 0.01^{b}	20	0.42 ± 0.01^{b}	20
ICG12988	0.48 ± 0.01^{c}	20	0.37 ± 0.01^{b}	17
ICG12991	0.47 ± 0.01^{c}	20	0.41 ± 0.02^{b}	13

Means within a column followed by a different letter are significantly different ($P<0.05$; ANOVA with contrasts). S.E. is the standard error of the mean and n represents the number of replicates.

Table 2 4: The mean values (±S.E.) for nymphal development time in days (D) and fecundity in time D (Md) for *A. craccivora* on 6 varieties of groundnut at different ages (7 DAP and 28 DAP). Aphids were collected from a Ugandan (a) and from a Malawian population (b)

a)

Ugandan aphid population

	Plants 7 DAP		Plants 28 DAP	
Variety	**D±S.E. (n)**	**Md±S.E. (n)**	**D±S.E. (n)**	**Md±S.E. (n)**
CG7	5.1 ± 0.1^{a} (20)	67.6 ± 1.5^{a} (20)	5.9 ± 0.1^{a} (17)	59.6 ± 2.7^{a} (17)
ICG SM90704	5.0 ± 0.0^{a} (20)	67.3 ± 1.3^{a} (20)	5.7 ± 0.1^{a} (20)	58.5 ± 3.1^{a} (20)
JL24	5.2 ± 0.1^{a} (20)	71.4 ± 1.4^{a} (20)	6.2 ± 0.2^{a} (17)	58.6 ± 2.0^{a} (17)
ICG5240	5.3 ± 0.1^{b} (20)	54.5 ± 2.5^{b} (20)	6.6 ± 0.2^{b} (15)	31.7 ± 2.7^{b} (15)
ICG12988	5.7 ± 0.1^{c} (20)	30.9 ± 2.3^{c} (20)	7.3 ± 0.3^{c} (10)	24.1 ± 2.8^{b} (09)
ICG12991	5.7 ± 0.1^{c} (20)	31.3 ± 2.1^{c} (20)	7.2 ± 0.3^{c} (09)	25.8 ± 3.7^{b} (08)

b)

Malawian aphid population

	Plants 7 DAP		Plants 28 DAP	
Variety	**D±S.E. (n)**	**Md±S.E. (n)**	**D±S.E. (n)**	**Md±S.E. (n)**
CG7	5.1 ± 0.1^{a} (20)	69.0 ± 1.4^{a} (20)	6.1 ± 0.1^{a} (19)	64.1 ± 3.0^{a} (17)
ICG SM90704	5.1 ± 0.1^{a} (20)	67.4 ± 1.0^{a} (20)	6.0 ± 0.1^{a} (18)	68.3 ± 3.2^{a} (15)
JL24	5.2 ± 0.1^{a} (20)	68.7 ± 1.4^{a} (20)	5.9 ± 0.1^{a} (19)	63.3 ± 2.5^{a} (18)
ICG5240	5.3 ± 0.1^{b} (20)	58.1 ± 3.0^{b} (20)	6.4 ± 0.1^{b} (20)	36.5 ± 2.0^{b} (20)
ICG12988	5.5 ± 0.1^{c} (20)	35.8 ± 2.0^{c} (20)	6.6 ± 0.1^{b} (19)	26.7 ± 2.5^{c} (17)
ICG12991	5.5 ± 0.1^{c} (20)	33.0 ± 2.1^{c} (20)	6.4 ± 0.1^{b} (17)	33.8 ± 2.4^{b} (13)

Means within a column followed by a different letter are significantly different ($P<0.05$; ANOVA with contrasts). S.E. is the standard error of the mean and n represents the number of replicates.

2.3.1.2. Survival of aphid nymphs on groundnut

The survival of aphid nymphs of both aphid clones (Uganda and Malawi), on plants (7 DAP) of all groundnut varieties tested was almost 100% (Table 2.5.). However, survival was significantly less on seedlings of ICG12991 and ICG12988 for the Ugandan aphid clone ($P<0.01$; Logit analysis). On older plants (28 DAP), the percentage survival of aphids was also almost 100% on CG7, ICG-SM90704 and JL24 but 92-94 % on ICG5240 ($P<0.01$) and 40-60% on ICG12988 and ICG12991 ($P<0.001$). Differences in aphid survival between the Ugandan and Malawian populations were observed on ICG12988 ($P<0.01$; Logit analysis). Both aphid colonies were however tested independently of each other and therefore caution is needed when making direct comparisons between both aphid populations.

Table 2 5: Survival (%) of nymphs after 5 days of two aphid clones (Uganda and Malawi) of *A. craccivora* on 6 varieties of groundnut at different plant ages (7 DAP and 28 DAP).

	Plants 7 DAP		Plants 28 DAP	
Variety	**Ugandan aphid clone**	**Malawian aphid clone**	**Ugandan aphid clone**	**Malawian aphid clone**
CG7	100^{a}	100^{a}	98^{a}	99^{a}
ICG SM90704	100^{a}	100^{a}	99^{a}	100^{a}
JL24	100^{a}	100^{a}	100^{a}	99^{a}
ICG5240	100^{a}	100^{a}	92^{b}	94^{b}
ICG12988	95^{b}	99^{a}	43^{c}	59^{c}
ICG12991	98^{b}	98^{b}	48^{c}	57^{c}

Mean nymphal survival (%) between varieties followed by a different letter are significantly different ($P\leq0.02$; Logit analysis).

2.3.1.3. Weight of adult aphids that had developed on groundnut

Aphids that had developed on ICG12991 (10 DAP) were lighter than those developed on other varieties ($P<0.001$; ANOVA). The mean weight for adults (n=25) that had developed on ICG12991 was 0.6 ± 0.02mg compared to approximately 1mg for the adults that had developed on JL24 (1 ± 0.2mg), CG7 (0.98 ± 0.02mg) and ICG SM90704 (0.98 ± 0.03mg).

2.3.2. Choice experiments

2.3.2.1. Aphid distribution on plants of two varieties

On JL24, adult apterous aphids generally moved from the older leaves and parts of the leaves to the younger parts of the plants of JL24 (Figure 2.2 a,c). The preferred feeding site on JL24 after 24h, was the petiole (42%). However, on ICG12991 aphids moved away from the petioles and leaf tissue and 82% settled on the side branches of the stem (SB) (Figure 2.2 b,d). Only a few aphids were generally present on older leaf tissue of both varieties after 24h (Figure 2.2 c,d).

Aphid nymphs also showed a preference for the petioles (36%), side branches (36%) and the buds (16%) as observed after 96h on JL24 (Figure 2.3 c). On ICG12991, nymphs finally settled as measured after 96h on the buds (42%) and side branches (41%) (Figure 2.3 d).

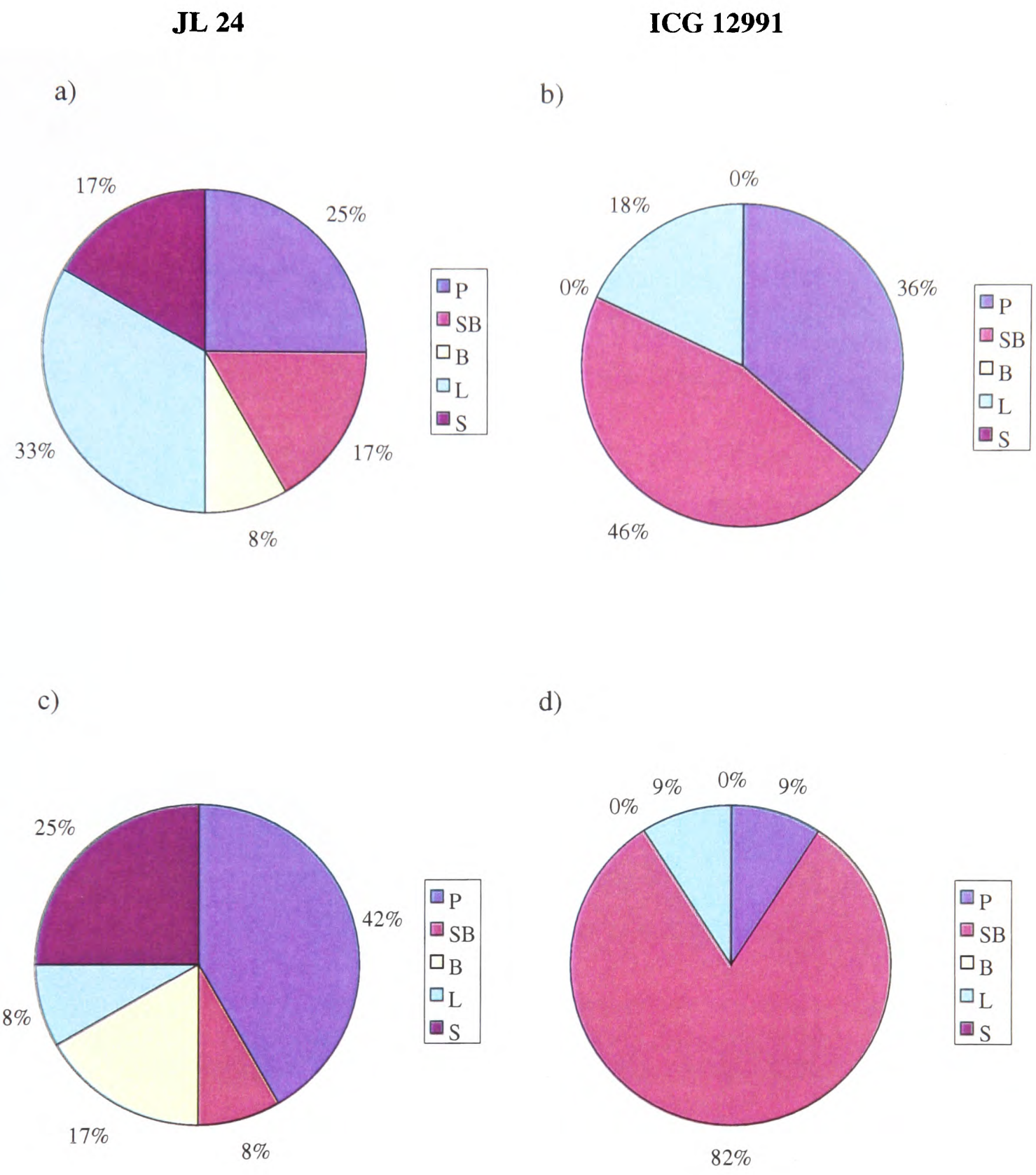
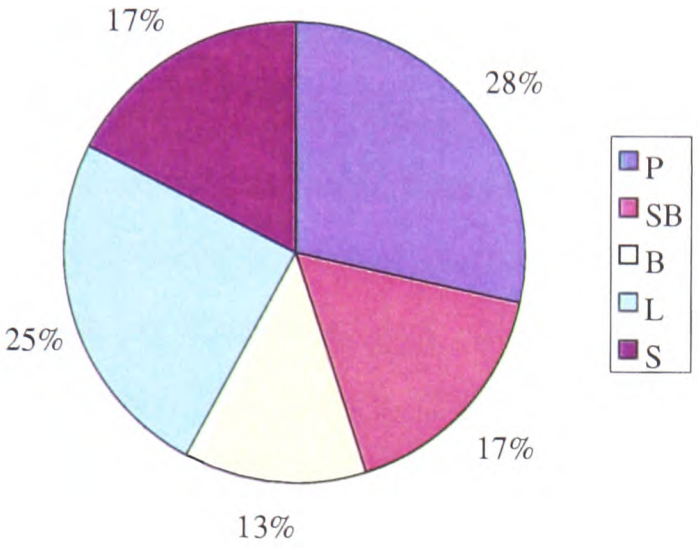


Figure 2 2: Within plant distribution (14 DAP) of adult apterous aphids on JL24 and ICG12991 after 6 hours (a,b) and 24 hours (c,d). P= Petiole, SB= Side branch of the stem, B= Bud, L= older leaf tissue, S= Main stem; n=12

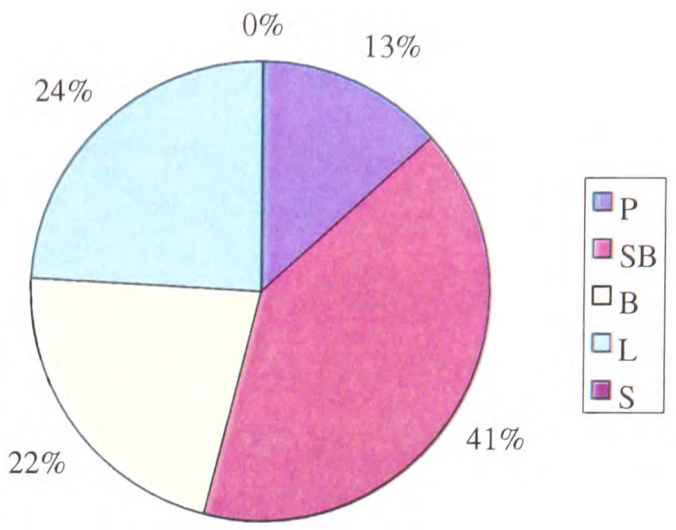
JL 24

ICG 12991

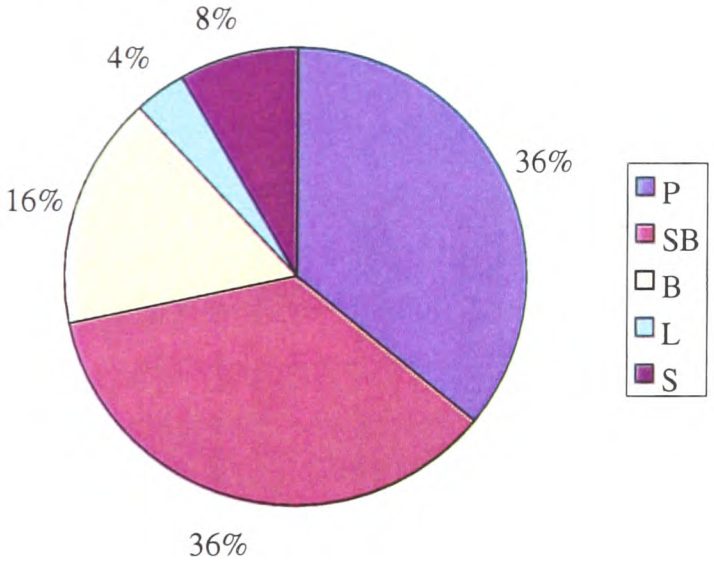
a)



b)



c)



d)

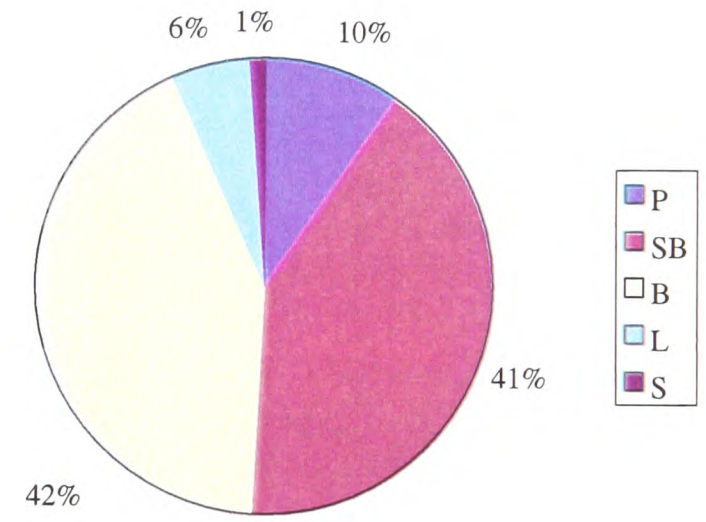


Figure 2 3: Within plant distribution (14 DAP) of aphid nymphs on JL24 and ICG12991 after 24 hours (a,b) and 96 hours (c,d). P= Petiole, SB= Side branches of the stem, B= Bud, L= older leaf tissue, S= Main stem; n=12

2.3.2.2. Aphid distribution between plants of two varieties

The aphids dispersed quickly around the arena and all insects had located a plant within 15 minutes of release. Within the first hour of the experiment significantly more aphids were on JL24 compared to ICG12991 ($P<0.01$; ANOVA), both when plants were 10 DAP and 28 DAP, and within 3 hours when plants were 17 DAP. Figure 2.4 shows the data of aphid distribution between plants of different groundnut varieties at 17DAP but the same trend was observed for the other two plant ages. The proportion of aphids on ICG12991 decreased gradually over 24 hours until only 10% of the aphids had finally settled on this variety. The proportion of aphids recovered on the tape surrounding the edges of the experimental set-up and numbers of aphids missing increased slightly with time. At the end of the experiment the numbers of nymphs were counted. The total number of nymphs per replicate for the different treatments was not significantly different, suggesting similarity in the aphids used. Figure 2.5 shows the total number of nymphs of all replicates counted on the plants.

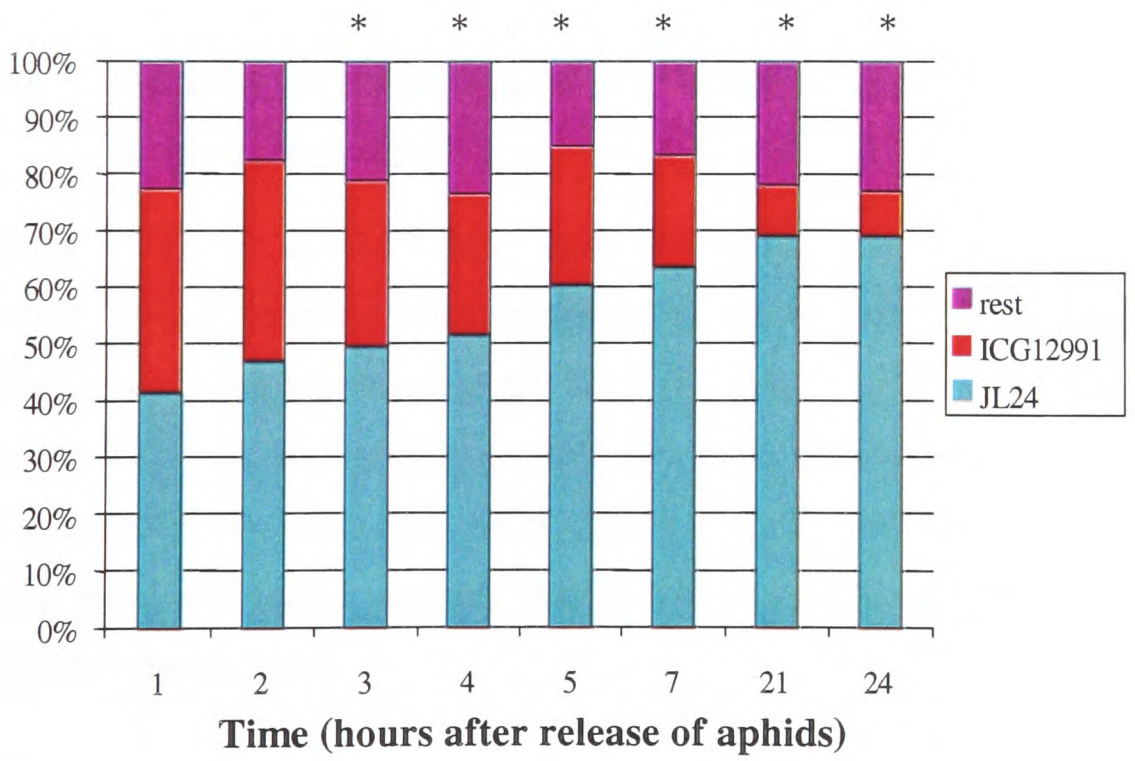


Figure 2 4: Between plant distribution (%) of aphids on JL24 and ICG12991 (17 DAP) at different times after release of 20 aphids/replicate (n=10-14). Aphids walking around or caught on the sticky tape on the edge of the arena are defined as 'rest'; * indicates significant differences of aphids on both varieties at $P<0.01$; ANOVA.

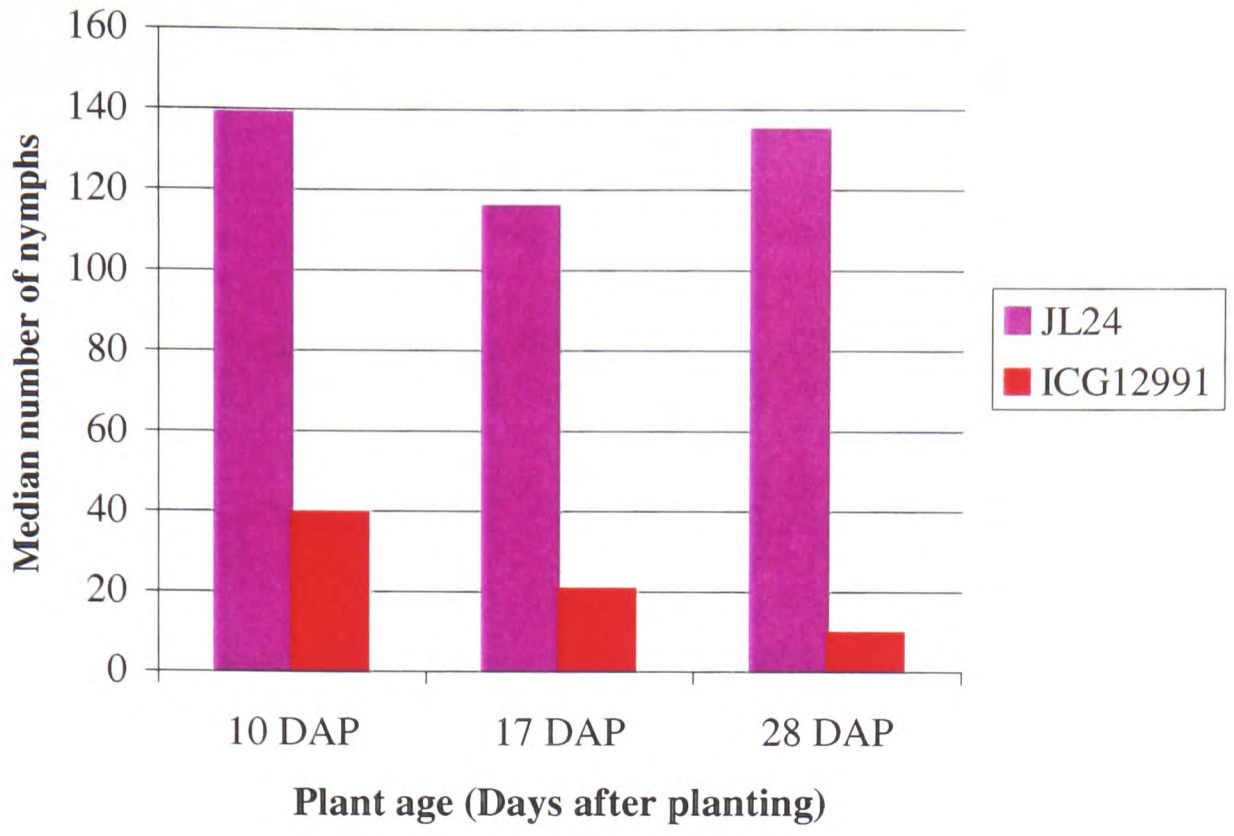


Figure 2 5: Number of nymphs (median) counted on groundnut plants after release of 20 apterous aphids in a choice experiment. Plants were 10, 17 and 28 DAP.

2.3.2. Additional screening for vector resistance on varieties originating from India and China.

Varieties that were collected from India and China were considered to be moderately resistant to *A. craccivora* when compared to known susceptible and resistant varieties (Table 2.6). Nymphal development time (D) and fecundity (Md) varied between the varieties and were all significantly different from the susceptible controls (JL24, CG7 and ICG-SM90704) except D on ICG9723. ICG11649 showed strongest resistance among the newly tested varieties (Rm = 0.42) and ICG SM99540 was as resistant as ICG12991 (Rm = 0.37).

Table 2 6: Mean values for nymphal development time in days (D ± S.E.), fecundity (Md ± S.E.) and intrinsic rate of increase (Rm ± S.E.) of *A. craccivora* on a range of varieties collected from India and China (21 DAP). Number of replicates was 15.

Variety	D	Md	Rm
JL24	5.1 ± 0.1^{a}	63.7 ± 1.6^{a}	0.60 ± 0.01^{a}
CG7	5.2 ± 0.1^{a}	60.4 ± 2.0^{a}	0.58 ± 0.01^{a}
ICG SM90704	5.1 ± 0.1^{a}	59.7 ± 2.2^{a}	0.60 ± 0.01^{a}
ICG9723	5.3 ± 0.1^{a}	45.7 ± 1.2^{b}	0.54 ± 0.01^{b}
ICG11735	5.8 ± 0.2^{b}	50.2 ± 2.9^{b}	0.50 ± 0.01^{c}
ICG11788	5.8 ± 0.2^{b}	49.4 ± 3.2^{b}	0.50 ± 0.01^{c}
ICG11649	6.2 ± 0.2^{c}	33.1 ± 2.3^{c}	0.42 ± 0.01^{d}
ICG99540	6.4 ± 0.1^{c}	25.0 ± 1.9^{d}	0.37 ± 0.01^{e}
ICG12991	7.0 ± 0.0^{d}	32.3 ± 2.2^{c}	0.36 ± 0.01^{e}

Means within a column followed by a different letter are significantly different ($P<0.05$; ANOVA with contrasts)

2.4. Discussion

The evolution of parthenogenesis in aphids has been of great importance in their population structure and in their characteristic high rates of natural increase (Rm), compared with other insects (Dixon, 1989). To measure Rm of *A. craccivora* on groundnut plants, the enclosure of the youngest plant parts in a perforated crisp bag was a reliable technique causing a minimal amount of disturbance to the aphids and enabled the recovery of dead aphids. Aphid development time, fecundity and therefore the Rm were reduced for both the Ugandan and the Malawian aphid clones on ICG5240, ICG12988 and ICG12991, irrespective of plant age. The effects of plant age were not directly compared, but Rm values were consistently higher for aphids feeding on seedlings due to both a shorter development time and an increased fecundity. Although intrinsic and extrinsic factors have a major influence on the Rm, aphids were reared under the same conditions of temperature, light and humidity and the adults that were used to generate first instar nymphs were similar in size and age. Therefore it was assumed that the weights of the first instar nymphs were also similar at the start of the Rm experiments and that the differences in aphid development and fecundity were due to differences in the varieties tested.

In addition to development time and reproduction, the intrinsic rate of increase is also affected by the survival of the immature and adult stages. Survival of nymphs on ICG12991 and ICG12988 was reduced when plants were older and therefore the Rm could not be calculated for each aphid on these varieties. In contrast, nymphal survival on seedlings of ICG12991 and ICG12988 were similar to those on the other varieties. The preference of *A. craccivora* for younger plants in the field (Booker, 1963; A`Brook, 1964; Farrell, 1976b) was reflected in these observations. Nevertheless, aphids were able to survive on the variety showing the strongest resistance, ICG12991 but survivors suffered a subsequent weight loss of 40% at adulthood. As a result, smaller first instar nymphs are likely to be produced in the next generation and take even longer to develop into adults (Dixon, 1985b; Dixon *et al.*, 1982). Also, smaller aphids are less likely to survive poor conditions than larger ones (Dixon, 1985a), which would be important when considering aphid performance under field situations.

The methodology used to assess aphid performance on groundnut plants was quick and reliable. Furthermore, additional screening of varieties of potential interest resulted in the identification of at least 5 more aphid resistant varieties collected by ICRISAT from India and China. Although the calculation of the Rm of an aphid population was greatly simplified by Wyatt & White (1977), it would be a useful tool for comparing the rate of increase among aphid populations of the same or different species on the same or different host plants. Unfortunately, no consensus has been reached yet and different research groups still apply different ways to calculate the values of Rm, which makes comparisons complicated. This way, the Rm of different clonal populations of *A. craccivora* was estimated at 0.3 on a susceptible cowpea variety (Annan *et al.*, 1997), which is much lower than the Rm found on susceptible groundnut varieties. Based on the published data, no conversion could be made and therefore aphid performance on cowpea and groundnut could not be compared. Also, the temperature in which the experiments on cowpea were conducted were lower (24 ± 4 °C) than the one used in this study (29 ± 1 °C) and could have contributed to lower Rm values.

To further investigate at what stage of the host plant selection process (see Figure 1.13) ICG12991 is rejected, choice experiments are of major importance. When given a choice, aphids quickly abandoned ICG12991 irrespective of plant age. Preliminary observations that led to the final design of the choice test showed an initial equal distribution of aphids on both JL24 and ICG12991. In a short period of 1 to 3h aphids rejected ICG12991 to settle on and colonise JL24. The effect of short-range volatile chemicals and leaf surface characteristics cannot be excluded and more experiments were conducted to evaluate these effects (Chapter 3, 5).

Based on the observations reported here, a resistance factor in ICG12991 is expressed at an early stage resulting in a quick rejection of the plants by the aphids and subsequent reduction in survival and performance of their offspring. General effects of plant ageing contributed to an even stronger degree of resistance. For example, an increasing distance between the epidermis and vascular bundles when plants grow older could lead to less successful infestations of aphids (Schnorbach, 1983; Klingauf, 1989). Nevertheless, the observed effects need to be assessed under field conditions because it

is well known that environmental factors such as temperature and rainfall, have a significant influence on aphid populations (Panda & Khush, 1995), which could interact with inherent plant resistance. Temperatures of 40 °C and heavy periods of rain could increase development time and mortality. Padgham *et al.*, (1990b) already illustrated an 86% reduction in aphid populations on the resistant groundnut variety ICG5240 as a result of rain compared to 60% on a susceptible variety.

The classification of plant resistance mechanisms into antibiosis and antixenosis (Painter 1951; Kogan & Ortman, 1978) was considered not to be helpful in understanding the underlying resistance mechanism in ICG12991. Based on the results presented here and in Padgham *et al.*, (1990b), different mechanisms are likely to operate in the aphid-resistant varieties ICG5240 and ICG12991. There was no initial rejection of ICG5240 by apterous aphids in a choice test (Padgham *et al.*, 1990b) and mortality was not significant. Also feeding was inhibited and interrupted on this variety, which would imply antibiosis as the resistance mechanism. In contrast to ICG5240, aphids quickly rejected plants of ICG12991 under laboratory conditions and aphid mortality was high compared to those on other varieties implying that antixenosis is the operating resistance mechanism in ICG12991.

The next step in the investigation was to confirm that high levels of aphid resistance are expressed under field conditions and that vector resistance is related to the lower incidence of groundnut rosette disease (Padgham *et al.*, 1990b; Chiyembekeza *et al.*, 1997; Merwe van der & Subrahmanyam, 1997; Subrahmanyam *et al.*, 1998; Naidu *et al.*, 1999b).

CHAPTER 3

HOST PLANT RESISTANCE IN GROUNDNUT AND PERFORMANCE OF *APHIS CRACCIVORA* UNDER GLASSHOUSE AND FIELD CONDITIONS IN SUB-SAHARAN AFRICA

3.1. Introduction

Groundnut rosette disease (GRD) is only efficiently transmitted in the field by *Aphis craccivora*, Koch (Homoptera, Aphididae), in a persistent and circulative manner (Storey & Bottomley, 1928; Storey & Ryland, 1955). Viruliferous immigrants alight within the field and establish primary sources of infection, especially when plants are widely spaced (Kennedy *et al.*, 1961). Once these infection sites are established, secondary spread of the disease can rapidly lead to infections throughout the crop (Thresh, 1983a). The nature and pattern of the disease spread can be influenced by plant age, crop density, timing and efficiency of transmission by viruliferous aphids, proximity to the source of primary infections, climatic factors, and natural enemies of vector populations within the crop (Evans, 1954; Booker, 1963; Farrell, 1976a,b).

Control strategies to GRD have been usually aimed at reducing vector populations to delay the onset and spread of the disease (Naidu *et al.*, 1999b; Chapter 1). However, such strategies are usually unsuccessful because of labour constraints and costs, the sowing sequence of crops and crop priorities and a lack of adequate resources. Therefore, resistance breeding is perceived as the most practical solution for rosette disease management.

Sources of GRD-resistance were first identified in varieties from West Africa in 1954 (Sauger & Cathérinet, 1954 a,b). The resistance was only partial against *Groundnut rosette umbravirus* and satellite RNA (GRV +satRNA) (Bock *et al.*, 1990) and the

varieties were completely susceptible to *Groundnut rosette assistor virus* (GRAV) (Olorunju *et al.*, 1991; Subrahmanyam *et al.*, 1998). The GRV +satRNA resistant varieties have been used in breeding programmes and a screening technique to evaluate global germplasm for GRD resistance was developed (the infector row technique; Bock, 1987). However, the process is labour intensive and does not allow recognition of GRAV resistant lines because, by itself, GRAV does not express disease symptoms in groundnut plants. The screening and breeding programmes were mainly focused on virus resistance irrespective of vector resistant varieties identified in Tanzania (Evans, 1954).

The potential of vector-resistant varieties in GRD control strategies is regaining attention only recently after vector-resistance was identified in a promising high yielding short duration variety ICG12991 (Mewe van der *et al.*, 2001). In Chapter 2 it has been shown that under laboratory conditions aphid performance and survival were significantly reduced on ICG12991 and when aphids were exposed in two-variety choice-tests, they quickly rejected this variety. It is well established that the level of plant-resistance to pests and diseases can be influenced by environmental factors and therefore, these effects on groundnut resistance to *A. craccivora* needed to be assessed under field conditions (Réal, 1953; Dixon, 1985a,b). Two visits were undertaken to Uganda in May 2000 and to Malawi in January 2001 to evaluate aphid resistance in groundnut varieties of potential interest, including ICG12991. In Uganda, trials were designed around the Serere Agricultural and Animal Production Research Institute's screening trials (SAARI) to investigate aphid infestation in the groundnut crop. At the experimental site of the International Crop Research Institute in the Semi-Arid Tropics (ICRISAT) based at Chitedze, Malawi, a different design to investigate aphid infestations was applied because there, aphids are artificially introduced in the experimental fields according to the infector row technique (Bock, 1987). The technique ensured that no varieties of interest escaped aphid infestation and a sampling procedure was developed to measure aphid population development on groundnut.

3.2. Materials and Methods

3.2.1. Uganda (May 2000)

The experimental sites were located around the Serere Agricultural and Animal Production Research Institute (SAARI) in Serere, Uganda (1° 31'N 33° 28'E), in collaboration with Dr. A. Chiyembekeza. Experiments in Uganda were mainly focused on aphid numbers under natural aphid infestation on selected varieties. Field and glasshouse experiments were designed to complement those that were described under controlled conditions at NRI, UK (Chapter 2).

3.2.1.1. Environmental conditions

Rainfall and temperature readings were collected from the SAARI meteorological station. Rainfall in the field plots was additionally measured using 4 rain-gauges which were placed over every alternate plot. Readings were taken every 24h.

3.2.1.2. Screening of groundnut varieties for *A. craccivora* under natural infestation

The experiment was designed in collaboration with Dr. Frances Kimmins (NRInt.-UK) and Mr. Bill Page (NRI-UoG-UK).

Ten groundnut varieties were screened for aphid numbers over a 10-day recording period. Each variety obtained from the ICRISAT screening programme was allocated a number which was preceded by ICG or ICG SM. Red Beauty and CG7 are improved varieties and used as susceptible controls because they are known to be completely susceptible to both the aphid vector and all three virus agents of GRD. A third susceptible control, Erudurudu, was used; this is widely grown by farmers around SAARI. ICG12991 was described as aphid resistant (Chapter 2) and results from ICRISAT-Malawi had shown that ICG SM90704 was GRV +satRNA resistant but aphid susceptible (Naidu *et al.*, 1999b). The varieties were labelled from A to J and until final data analysis it was not known which variety was linked with which letter (Appendix 2a).

Four replicates of 10 varieties were planted in a randomised block design on April 17th 2000 (Table 3.1). Each plot consisted of 6 rows of groundnut plants of the variety of interest, 5m long and 45cm apart. A uniform spacing of 45cm was maintained between the replicates and a guard row of the aphid susceptible ICG SM90704 was planted around the trial, 60cm away at the sides and 30cm at the ends to minimise edge effects (Appendix 2b).

Twenty plants per variety per replicate were randomly sampled for aphid numbers with the assistance of Dr. Frances Kimmins and Mr. Bill Page. Aphid counts were carried out every other day for 10 days, so that 4000 plants in total were sampled. Plant age was approximately 21 days after planting (DAP) at the start of the first recordings and for each variety a mean percentage of germination was calculated in relation to the number of seeds planted. Groundnut rosette disease incidence (%), based on symptom expression, was obtained by calculating the number of diseased plants in relation to total number of germinated seeds. At the end of the growing season the number of days from planting the groundnut seeds till harvest and the yield, expressed as weight of dry pods (Kg) were obtained.

Table 3 1: The arrangement of the field plots at SAARI- Uganda. Varieties are labelled A-J and for those with their accession number in brackets, the full designations are preceded with ICG(SM).

Replicate 1	Replicate 2	Replicate 3	Replicate 4
Variety/ Accessions			
J (Igola 2) Virus resistant	D	C	B
I (93557)	A	E *	G
H (94584)	B	F	H
G (Red Beauty) Susceptible control	C	D *	A
F (12991) Aphid resistant	G	B	J
A (93530)	H	G *	I
B (93535)	I	H	D
C (93524)	E	A *	C
D (94581)	F	J	E
E (99540)	J	I	F

*: Position of the rain-gauges

3.2.1.3. Performance of apterous *A. craccivora* on 3 groundnut varieties in a no-choice experiment

Aphid performance tests were conducted after an aphid colony was successfully established in the glasshouses on a susceptible, local groundnut variety (var. Erudurudu). The experiment was designed in response to data obtained on aphid performance on groundnut under laboratory conditions at NRI (Chapter2). In a separate field as the one used for 3.2.1.2, the test plants were selected over two rows per variety and three varieties were selected for the experiment (Appendix 3). These varieties were ICG SM90704 (GRD resistant and aphid susceptible), ICG12991 (GRD susceptible and aphid resistant) and CG7 (GRD and aphid susceptible). Each plant was covered with two crisp bags (Cryovac Europe, St.Neots, UK), the first one to cover the top of the plants containing single apterous aphids and the second one to cover the whole plant to prevent natural aphid infestation and to protect the experimental set-up from splashing mud during periods of heavy rainfall. The covered plants were additionally protected from the heavy rain and mud by polystyrene tiles. The adults were caged on the plants for 6 days, after which the tiles and bags were removed (n=10). The top of the plant was cut and immersed in 70% alcohol in a vial. The number of nymphs and adults on each plant was then counted in the laboratory. Data on numbers of insects were analysed by non- parametric statistics with multiple comparison (Kruskal-Wallis).

3.2.1.4. Performance of apterous *A. craccivora* on 3 groundnut varieties in a choice experiment

Data from the laboratory at NRI (Chapter 2) indicated that aphids quickly rejected ICG12991. A choice-test was conducted to assess this observation in the field. One adult apterous aphid from the glasshouse culture was placed on test plants of three varieties, ICG SM90704, ICG12991 and CG7, which were also used in the no-choice experiment (3.2.1.3). In total, 18 plants per variety (2 x 9), free from aphids, were marked in the field and inspected for colonies 48h after introduction of one adult apterous aphid on each plant. The plants were left naturally without protection from ambient conditions. The varieties were planted in plots of 6 rows and 5 metres long. The first replicate was set-up on 13th May 2000 and the second on 15th May 2000. The

number of nymphs per colony and number of colonies on each variety was counted and analysed with non- parametric statistics (Kruskal-Wallis).

3.2.1.5. Performance of apterous *A. craccivora* on seedlings of ICG12991 in the glasshouse

It was established in the laboratory at NRI (Chapter 2) that aphids survived well on seedlings (7 DAP) of ICG12991 but not on older plants of this variety (28 DAP). This observation was tested by placing one adult apterous aphid from the culture (var. Erudurudu) on seedlings of ICG12991. The plants were screened for colonies 48h later (n=34). As a control, the aphid susceptible variety Erudurudu was included (n=17). The plants were covered with crisp bags to avoid natural infestation and aphids walking off the plants. Survival was calculated as the number of living adults that could be recovered on the plants after 48h.

It was also observed that aphid colonies could not be established on a susceptible groundnut variety Erudurudu when the aphids were collected from another legume, cowpea. Therefore a similar experiment as described in previous paragraph was conducted. Aphids were collected from weed plants in the field and confined on seedlings of ICG12991 and Erudurudu (n=10). Aphid survival and their offspring were recorded after 24h.

The weed species could not be identified but aphids were abundant on this species in and around the field plots.

3.2.2. Malawi (January 2001)

The main objective for this field trip was to develop an aphid screening method on groundnut. Two short duration groundnut varieties of interest, ICG12991 and ICG SM99540, and a susceptible control JL24 were assessed for aphids under high pressure of viruliferous aphids using the infector row technique (Bock, 1987). The fields were located on the GRD screening sites at ICRISAT (13° 58'S 33° 49'E).

When conducting glasshouse experiments, the glasshouses were equipped with a desert cooling system and the roof was covered to keep temperatures down.

3.2.2.1. Environmental conditions

Rainfall data were collected from the ICRISAT meteorological centre. Due to missing data points a different type of graph is presented than the one used to describe the environmental conditions in Uganda. No recordings of temperature in the field were available.

3.2.2.2. Screening of groundnut varieties under high pressure of viruliferous aphids

Selected groundnut varieties were screened for aphid colonies to assess aphid resistance under field conditions. Three varieties were screened for aphid colonies and colony size and 20 plants per variety per replicate were randomly screened. The plants were in the flowering stage (40 DAP) and additional data on plant height and number of growing points were recorded on the last day of sampling.

The field trial was designed in an 8x8 lattice and replicated three times. Sixty-four varieties were planted in plots of 3 rows x 6m x 0.6m, with seed spacing of 10cm and 200 seeds of each variety. A field plan is provided in Figure 3.1 and the numbers therein are conform those in Table 3.2. Aphid population recordings were conducted between 09th January 2001 and 18th January 2001. All three replicates for all 3 varieties were successfully sampled on 09th, 15th and 18th January 2001 but for the latter date, recordings were taken after the rains. On 11th January, only data from replicate 3 were collected prior to heavy rains. On 12th January two replicates were completely sampled

for JL24 and ICG12991, whereas all three replicates were sampled for ICG SM99540. Aphid colonies were counted and their location specified as on leaf versus flower tissue. A colony was considered to have formed when at least two aphids from any stage were present together. The number of aphids per colony was then ranked in an order of magnitude: 0 = no colony or single aphid (record nymph or adult), 1 = 2-10, 2 = 11-100, 3 = 101-1000, 4 = >1000. The number of plants containing at least one colony was calculated as a percentage of the number of plants counted. Also the proportion of colonies found on flower versus leaf tissue was calculated.

3.2.2.3. Performance of alatae morphs of *A. craccivora* on groundnut varieties in a no-choice experiment in the glasshouse

An experiment was conducted to confirm resistance to *A. craccivora* in ICG12991 under glasshouse conditions in Malawi (Chapter 2). A new variety of potential interest for future breeding programmes, ICG SM99540, was included in the experiment and JL24 was the susceptible control variety (3.2.1.2; P.J.A. van der Merwe pers. comm). One winged aphid (alate morph) per plant per variety was caged for 6 days and the number of offspring was then counted. Plants were 19-23 DAP and each plant was potted and enclosed in a crisp bag to contain the aphids. The plants were arranged in a randomised block design with 4 plants per variety in 4 blocks (n=16).

The experiment was repeated for plants aged 28-32 DAP and ICG SM907040 was included as an extra susceptible control apart from JL24 (n=7). The plants were arranged in a randomised design but this time three adult alatae were caged on the plants for 7 days. The number of nymphs on each variety was counted after 7 days and compared with non- parametric analysis with multiple comparisons (Kruskal-Wallis).

Guard rows of ICG-SM 90704

Replicate 1

1	2	3	4	5	6	7	8
9	10	11	12	13	14	15	16
17	18	19	20	21	22	23	24
25	26	27	28	29	30	**31**	32
33	34	35	36	37	38	39	40
41	42	43	44	45	46	47	48
49	50	51	52	53	54	55	56
57	58	59	60	61	62	**63**	**64**

Replicate 2

1	9	17	25	33	41	49	57
2	10	18	26	34	42	50	58
3	11	19	27	35	43	51	59
4	12	20	28	36	44	52	60
5	13	21	29	37	45	53	61
6	14	22	30	38	46	54	62
7	15	23	**31**	39	47	55	**63**
8	16	24	32	40	48	56	**64**

Replicate 3

1	10	19	28	37	46	55	**64**
9	2	51	44	61	30	23	40
17	50	3	36	29	62	15	48
25	52	35	4	21	14	**63**	56
33	58	27	20	5	54	47	16
41	26	59	12	53	6	39	24
49	18	11	60	45	38	7	32
57	34	43	52	13	22	**31**	8

F1 generations

Figure 3 1: Field plan showing the arrangement of the plots. Varieties linked with their number are presented in Table 3.2. Highlighted in bold are the labelled varieties that were screened for aphid colonies and numbers. Note that in between the plots, rows of infected plants were planted but not indicated in this figure (infector row technique (Bock, 1987).

Table 3 2: Varieties that were planted for groundnut rosette resistance screening trials at ICRISAT-Malawi. Highlighted in bold are the varieties that were screened for aphid numbers and colonies

Number	Variety	Number	Variety	Number	Variety
1	ICG SM99501	23	ICG SM99527	45	ICG SM99554
2	ICG SM99502	24	ICG SM99528	46	ICG SM99555
3	ICG SM99503	25	ICG SM99529	47	ICG SM99556
4	ICG SM99504	26	ICG SM99530	48	ICG SM99557
5	ICG SM99507	27	ICG SM99531	49	ICG SM99558
6	ICG SM99508	28	ICG SM99534	50	ICG SM99561
7	ICG SM99510	29	ICG SM99538	51	ICG SM99562
8	ICG SM99511	30	ICG SM99539	52	ICG SM99565
9	ICG SM99512	**31**	**ICG SM99540**	53	ICG SM99566
10	ICG SM99513	32	ICG SM99541	54	ICG SM99567
11	ICGSM99514	33	ICG SM99542	55	ICG SM99568
12	ICG SM99515	34	ICG SM99543	56	ICG SM99569
13	ICG SM99516	35	ICG SM99544	57	ICG SM99571
14	ICG SM99518	36	ICG SM99545	58	ICG SM99572
15	ICG SM99519	37	ICG SM99546	59	ICG SM99573
16	ICG SM99520	38	ICG SM99547	60	ICG SM99574
17	ICG SM99521	39	ICG SM99548	61	ICG SM99575
18	ICG SM99522	40	ICG SM99549	62	ICG SM99577
19	ICG SM99523	41	ICG SM99550	**63**	**ICG12991**
20	ICG SM99524	42	ICG SM99551	**64**	**JL24**
21	ICG SM99525	43	ICG SM99552		
22	ICG SM99526	44	ICG SM99553		

3.3. Results

3.3.1. Uganda (May 2000)

3.3.1.1. Environmental conditions

The rainfall and temperature measurements from the rain-gauges in the experimental plots and details on the sampling days are presented in Appendix 4a.

3.3.1.2. Screening of groundnut varieties for *A. craccivora* under natural infestation

The cumulative number of plants with at least one aphid during the sampling period and per sampling day is represented for each variety in Figure 3.2a,b. Three varieties, ICG12991 (F), ICG SM99540 (E) and ICG SM93535 (B) had less than 40 infested plants in total (10%), whereas the highest numbers of infested plants were found on ICG SM93524 (C, 21%) and Red Beauty (G, 20%) (Figure 3.2a). On the first sampling day (9^{th} May 2000) aphid-infested plants were generally low, probably because of the heavy rains on the 8^{th} and 9^{th} May 2000. Over time, the number of infested plants increased for all varieties except ICG12991 (Figure 3.2b). Less than 5 plants of this variety were infested over the sampling period and it also was the only variety where on some plants no aphid colonies were found. Results on rosette incidence, germination rates, days to harvest and yield are presented in Table 3.3. The local control variety Red Beauty had the highest rosette disease incidence (29.5%) and provides an indication of disease incidence in this location of Uganda. However, the rosette disease incidence on all other varieties was low irrespective of aphid abundance and was less than 1%. This was expected since the material was being tested for their rosette disease incidence.

Red Beauty, ICG12991 and ICG SM99540 were harvested less than 100 DAP compared to the other varieties, which were harvested after approximately 110 DAP. Yields (kg dry pods) were twice to three times higher for ICG12991 (2.6 kg ± 0.1) and ICG SM99540 (3.4 kg ± 0.3) compared to the local control variety Red Beauty (1.1 kg ± 0.1) and these varieties germinated very well (98-99%).

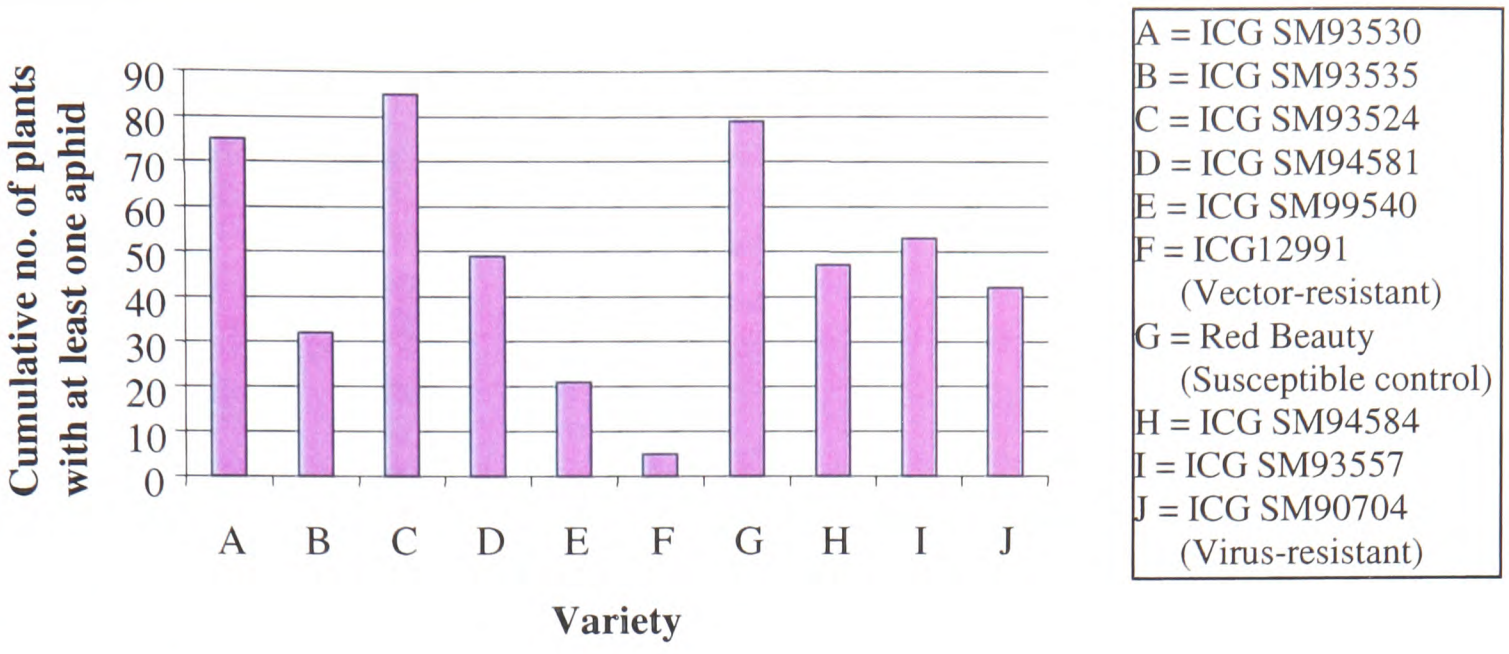


Figure 3 2: Cumulative number of plants with at least one aphid per sampling day at SAARI-Uganda. Total number of plants per variety sampled was 400 over 5 alternating sampling days.

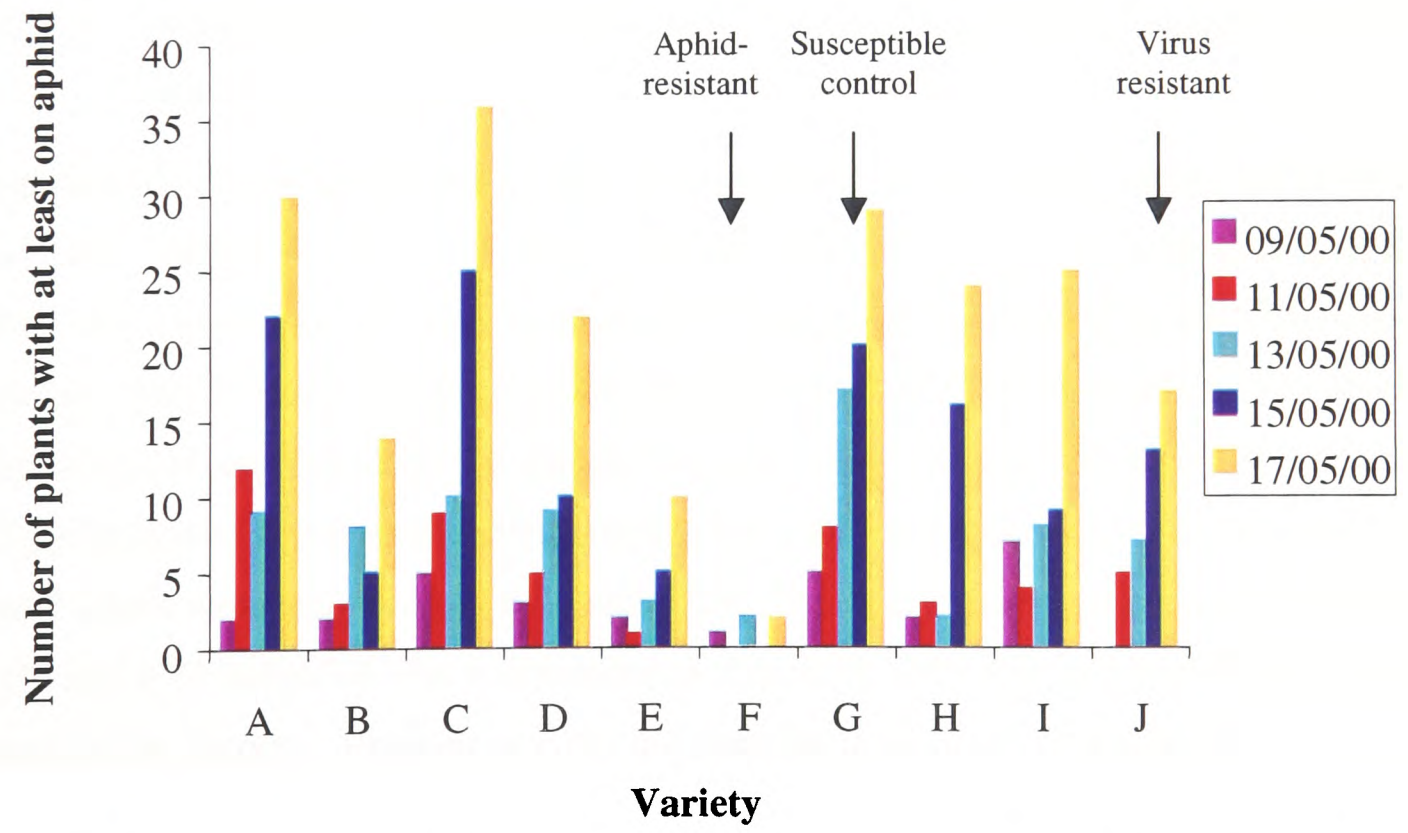


Figure 3.2b: Number of plants with at least one aphid per sampling day at SAARI-Uganda. Total number of plants sampled per variety per day was 80 (20 x 4 replicates).

Table 3 3: Evaluation of selected varieties for seed germination, days to harvest, rosette incidence and yield of dry pods in on-station trials at SAARI in May 2000.

Variety/ Accession	Germination (%)[1]	Days to harvest[2] (± S.E.)	Rosette incidence (%)	Yield: weight of dry pods (Kg)
ICG SM93530	95	108.5 ± 0.3	0.5	2.3 ± 0.5
ICG SM93535	76.3	109.3 ± 0.3	0	1.7 ± 0.2
ICG SM93524	97.5	108.3 ± 0.3	0	2.4 ± 0.1
ICG SM94581	93.8	110.5 ± 2.2	0	2.9 ± 0.2
ICG SM99540	97.5	98.5 ± 0.3	0.3	3.4 ± 0.3
ICG12991 (vector resistant)	98.8	98.6 ± 0.3	0.5	2.6 ± 0.1
Red beauty (Susceptible control)	98.8	98.0 ± 0	29.5	1.1 ± 0.1
ICG SM94584	92.5	110.8 ± 2.1	0.3	2.3 ± 0.3
ICG SM93557	67.5	106.8 ± 0.3	0.3	1.7 ± 0.1
ICG SM90704 (virus resistant)	90.0	111.3 ± 1.9	0	2.1 ± 0.1

[1]Germination was calculated as number of seeds planted in relation to number of germinated seeds
[2]Days to harvest is the number of days from planting the seeds till harvest

3.3.1.3. Performance of apterous *A. craccivora* on 3 groundnut varieties in a no-choice experiment

Significantly fewer adults and nymphs were counted on ICG12991 compared to CG7 and ICG SM90704 when one adult apterous aphid per plant was caged for 6 days, (P<0.001; Table 3.4). Number of adults and nymphs on CG7 and ICG SM90704 were similar. Only 2 adults aphids out of 10 were recovered on ICG12991 after 6 days which had produced approximately 10 nymphs in total. On CG7 and ICG SM90704, 52 and 43 nymphs per plant were recorded respectively. All ten adults were recovered on CG7 and 7 adults were recovered on ICG SM90704. The mean number of growing points on CG7 and ICG SM90704 was 6 compared to 5 on ICG12991 and the mean height of the plants of the former 2 varieties was 15 cm compared to 20 cm for the latter.

Table 3 4: Number of adults and nymphs on three groundnut varieties when one adult apterous aphid per plant was caged for 6 days. N=10

Variety	CG7 Susceptible. control	ICG SM 90704 Virus resistant	ICG12991 Aphid resistant
No. of adults (median)	9	4	0
No. of nymphs (median)	52	43	4

3.3.1.4. Performance of apterous *A. craccivora* on 3 groundnut varieties in a choice experiment

Rainfall was low on the days of this experiment ranging from 0-13 mm and minimising the risk of aphids being washed off the plants. The plants from the second replicate were screened for aphids prior to the heavy rains on the 17th May. No colonies were found on any of the 18 plants of ICG12991, 48h after single adult aphids were introduced on each plant, whereas colonies were found on seven plants of CG7 and on eight plants of ICG SM90704 (Table 3.5). The median number of nymphs per colony was 4 on CG7 and 3 on ICG SM90704 and was not significantly different (Mann-Whitney *U*-test). Details about the plant characteristics are also presented in the table.

Table 3 5: Aphid colonies and number of insects per colony* on three varieties in the field when single aphids were placed on the plants over 48h. N = 18 for each variety. Gps = growing points of the plant.

Variety	CG7 Susceptible. control	ICG SM 90704 Virus resistant	ICG12991 Aphid resistant
No. of colonies*	7	8	0
No. of nymphs per colony (median)	4	3	0
Plant height (median)	15	14	18
No. of gps per plant (median)	7	8	6

* : A colony represented at least 2 aphids of any stage together

3.3.1.5. Performance of apterous *A. craccivora* on seedlings of ICG12991 in the glasshouse

Aphids which were collected from the glasshouse culture on groundnut plants (var. Erudurudu) accepted young plants of ICG12991 and Erudurudu (8 DAP) more easily than those collected from other sources such as weed plants and cowpea[1] (Table 3.6). The median number of nymphs per colony was generally higher on variety Erudurudu. Twice as many nymphs were found on Erudurudu than on ICG12991 when aphids were collected from the culture but a similar number was found on both varieties when aphids were collected from weeds. However, caution is needed because aphids were given only 24h on groundnut when collected from weed plants compared to 48h when collected from groundnut. Nevertheless, aphids collected from weed performed less well on groundnut than when collected from groundnut.

Table 3 6: Aphid survival on seedlings of groundnut. Aphids were collected from the glasshouse groundnut culture (a) or collected from weeds in the field (b). n= number of replicates at the start of the experiment.

a)

Aphids collected from glasshouse groundnut culture (var. Erudurudu)

	48h access period on groundnut	
Variety	**ICG12991**	**Erudurudu**
% Survival	53	76
No. of nymphs per colony (median)	6	11
n	34	17

b)

Aphids collected from weed plants in the field

	24h access period on groundnut	
Variety	**ICG12991**	**Erudurudu**
% Survival	30	60
No. of nymphs per colony (median)	9	10
n	10	10

[1] Aphids were originally collected from cowpea to establish populations on groundnut but this failed

3.3.2. Malawi (January 2001)

3.3.2.1. Environmental conditions

The rainfall and temperature measurements from the rain-gauges in the experimental plots and details of the sampling days are presented in Appendix 4b.

3.3.2.2. Screening of groundnut varieties under high pressure of viruliferous aphids

On each sampling date, more plants of JL24 were infested with aphid colonies than ICG12991 and ICG SM99540 (Figure 3.3). An initial increase of infestation for all three varieties was followed by a marked reduction of infested plants of ICG12991 and ICG SM99540 on the third sampling day, whereas a 100% infestation was recorded on JL24. At the end of the sampling period, fewer infested plants were observed on all varieties but these last counts were performed after heavy rainfall (18th January 2001) (50mm).

The proportion of colonies on flower tissue was different for JL24, ICG12991 and ICG SM99540 (Figure 3.4). Only a low proportion (5%) of the colonies was found on the flowers of JL24 and this remained stable during the sampling period. By contrast, the proportion of colonies on the flowers of plants of ICG12991 was the highest on the second sampling day reaching a maximum of 71%. Also a higher proportion of colonies on the flowers of ICG SM99540 was observed and reaching a maximum of 35% on the first sampling day. For the latter two varieties the proportion declined strongly and at the last sampling day no colonies were found on the flowers of any of the varieties (18th January 2001).

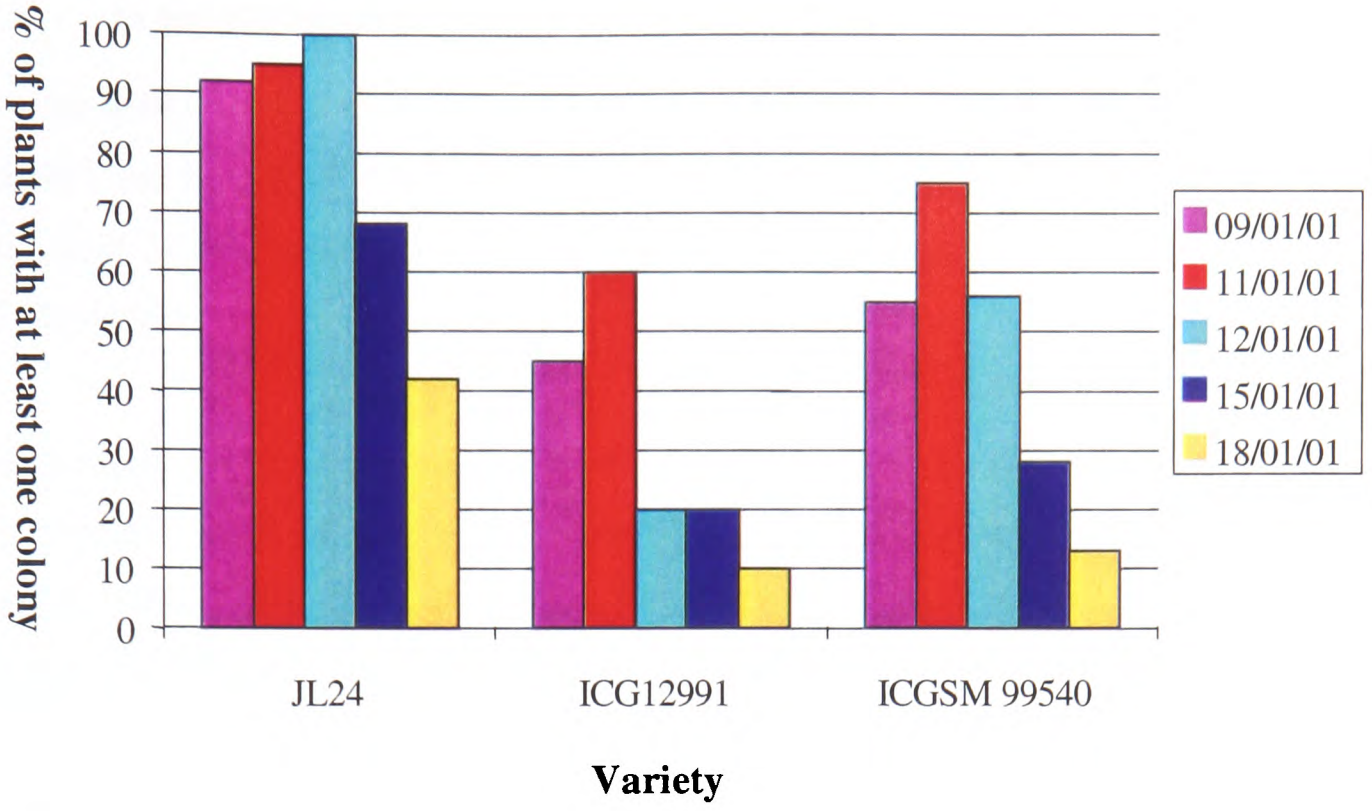


Figure 3 3: Percentage of plants of three varieties with at least one aphid colony under high pressure of aphids per sampling day at ICRISAT-Malawi. Total number of plants per variety sampled per day was 60.

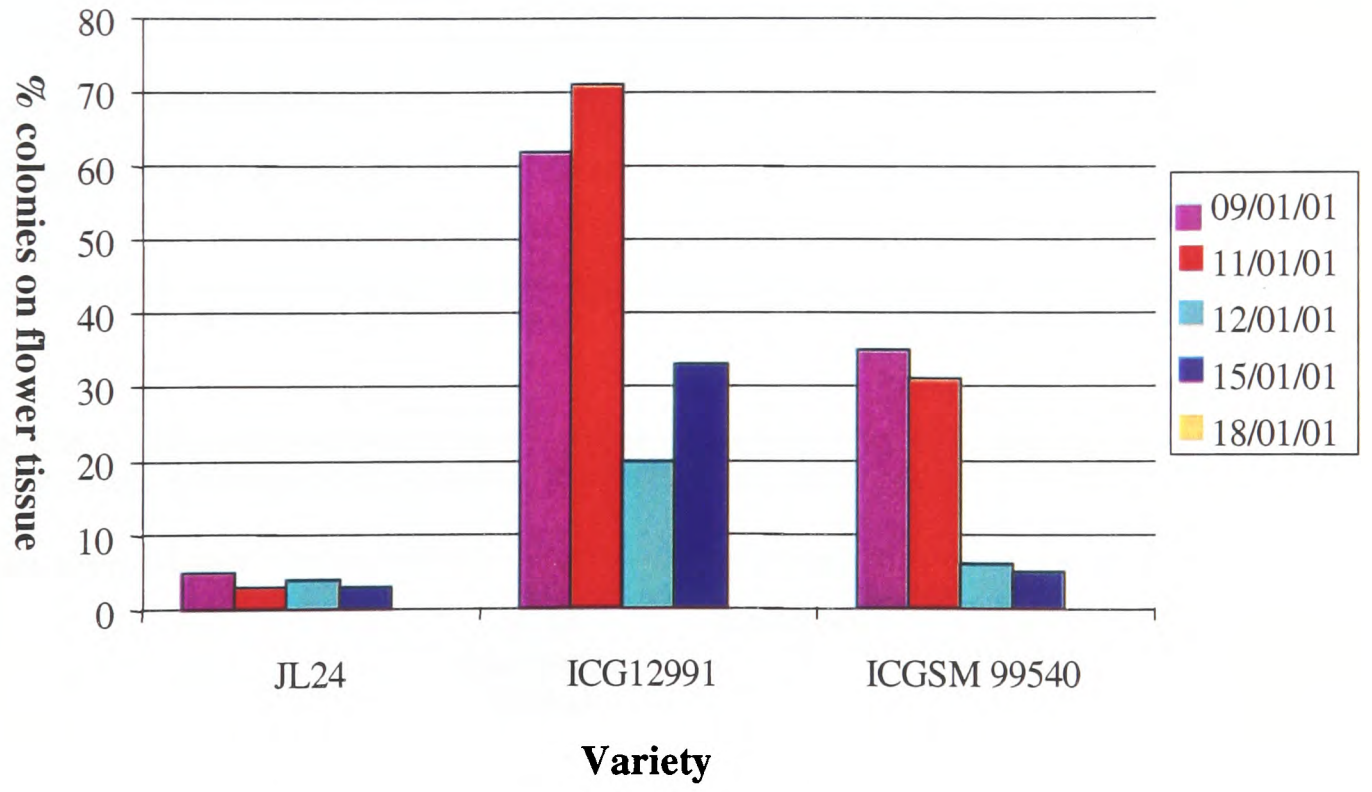


Figure 3 4: Proportion of colonies per sampling day found on flower tissue of three groundnut varieties. Total number of plants per variety sampled per day was 60.

3.3.2.3. Performance of alatae morphs of *A. craccivora* on groundnut varieties in no-choice experiment in the glasshouse

One alate aphid produced significantly more nymphs per colony on JL24 compared to ICG12991 and ICG SM99540 ($P<0.01$; Table 3.7a). After the 6-day period, 63% of the alatae could be recovered from the plants of JL24, 56% from ICG SM99540 and 38% from ICG12991.

When 3 alatae per plant per variety were caged for 7 days, significantly more nymphs were recorded on JL24 and ICG SM90704 than on ICG12991 and ICG SM99540 ($P<0.001$; Table 3.7b). On 2 plants out of 7 of ICG12991, no nymphs were found and a big range in the size of colonies was generally observed, 2-8 on ICG12991 and 19-147 on the other varieties.

Table 3 7a: Number of nymphs on different groundnut varieties when one alate aphid per plant was caged for 6 days; n=16 at start of experiment. Range of the size of the colony is also given

	JL24 Susceptible control	ICG12991	ICG SM99540
Nymphs per colony (median)	12	5	4
No. of aphids per colony (range)	6-28	3-7	2-6
n	13	7	9

Table 3.7b: Number of nymphs on different groundnut varieties when three alate aphids per plants were caged for 7 days; n=7 at start of experiment. Range of the size of the colony is also given

	JL24 Susceptible control	ICG12991	ICG SM99540	ICG SM90704 Susceptible control
Nymphs per colony (median)	83	5	20	93
No. of aphids per colony (range)	19-131	2-8	5-40	36-147
n	7	5	7	7

3.4. Discussion

Aphid resistance in ICG12991 under controlled conditions in the laboratory was described in chapter 2 and the field experiments confirmed that the resistance was robust in Uganda and Malawi. Under natural and artificial aphid pressure, fewer plants of ICG12991 were infested by aphids and it appeared that they could not establish colonies on this variety compared to other varieties including the virus resistant ICG SM90704. Natural conditions were used in Uganda and early planting at the appropriate densities could have reduced aphid alighting and primary infection (Kennedy *et al.*, 1961), but in Malawi the infector row technique ensured high aphid pressure in and around the groundnut plots.

Abiotic factors such as sunlight, temperature and rainfall play an important role in aphid population structure (Dixon, 1985a) but heavy rain spells could only partially account for the lower number of aphids on ICG12991 in Uganda. Although more aphids are likely to be washed off the resistant plants than susceptible plants (Padgham *et al.*, 1990b), the consistently low number of nymphs recorded in no-choice and choice experiments with adult aphids in the glasshouse, corroborated the reported laboratory results which showed that aphid survival and fecundity were significantly reduced on ICG12991. Consequently, aphid colonies and therefore population built-up and aphid spread within the crop varieties would be reduced as was observed in Malawi. Similar observations of aphid behaviour on ICG12991 were also made on ICG SM99540 which was identified as highly aphid resistant and therefore considered in future research at NRI, UoG.

The results showed that aphid resistance was now undoubtedly expressed in ICG12991 in the field as well as under laboratory and glasshouse conditions (Chapter 2). However, as older plants were used in Malawi, it was noted that flowers had been produced on all varieties and substantial colonies were present on flower stems including those of ICG12991. A significant proportion of all aphid colonies was found on the flowers of the aphid resistant varieties ICG12991 and ICG SM99540 compared to the aphid susceptible variety JL24. Aphids, which were washed off the plants and survived the heavy rain, were likely to re-colonise the tissues closest to the soil first, hence the high proportions on the flowers. However, the abundance of aphids on the

flower tissues led to the speculation that resistance in ICG12991 and ICG SM99540 may not be expressed in the flowers.

All the varieties that were screened in Uganda had a very low disease incidence compared to Red Beauty, a local grown variety which is susceptible to aphid and virus. It was assumed that alighting of viruliferous immigrants was equal for all varieties in the trials and that the spread of the disease was prevented because of the poor population development of aphids on the resistant varieties. In contrast, virus resistance was most likely to act in the varieties where aphid populations were building-up but with low recorded disease incidence. Partial resistance to GRV +satRNA was already established in ICG SM90704 but this partial resistance to the virus complex and high susceptibility to aphids would still allow transmission of GRAV on this variety (Robinson *et al.*, 1999). Plants only infected with GRAV do not show any symptoms (Murant *et al*, 1988) and rating of GRD incidence is based on symptom expression only (Bock, 1987). These issues will be discussed elsewhere but this is the first report that directly correlated vector resistance with low rosette disease incidence in the field. Detailed transmission studies were carried out to better understand the relation between the virus agents, the aphid vector and the groundnut host plant (Chapter 4).

The identification of vector resistance in at least two varieties and the relatively easy technique to screen varieties on aphid colonies would be an additional tool in identifying new GRD resistant material and to broaden the genetic basis of resistance to rosette disease. Hitherto, research failed to reveal varieties expressing both vector and virus resistance but this could be a new strategy to control rosette disease. These varieties would be especially useful because farmers usually fail to plant their crops early and in close densities to reduce primary infection by viruliferous aphids. Preliminary observations showed that aphids collected from weeds or cowpea plants did not perform well on the susceptible varieties of groundnut which could question the perpetuation of the disease by aphids migrating between different hosts (Adams & Farrell, 1967). Additionally, no alternative host for the virus agents has been found yet. Aphid resistance in ICG12991 was demonstrated in laboratory and field situations and the effect of this resistance on the transmission of the virus agents of groundnut rosette disease was further investigated (Chapter 4).

CHAPTER 4

TRANSMISSION OF THE GROUNDNUT ROSETTE VIRUS AGENTS BY *APHIS CRACCIVORA*

4.1. Introduction

Groundnut resistance to the aphid vector of *Groundnut rosette virus* disease, *A. craccivora*, was described in variety ICG12991 in laboratory and field studies (Chapters 2, 3). The variety is also known to be completely susceptible to all three virus agents of the disease, GRAV (*Groundnut rosette assistor virus*, Fam. Luteoviridae), GRV (*Groundnut rosette virus*, Fam. Umbraviridae) and satellite RNA (satRNA) (Merwe van der *et al.*, 2001). The satRNA was shown to be largely responsible for disease symptoms, either "chlorotic rosette" or "green rosette" (Hayes, 1932; Storey & Ryland, 1957; Gibbons, 1977) and has always been found together with GRV in nature (Murant *et al.*, 1988). Chlorotic rosette is ubiquitous in sub-Saharan Africa, whereas green rosette has been reported only from West African countries and from Uganda, northern Malawi and Angola.

Successful transmission of groundnut rosette disease by the aphid vector and, consequently, the survival of the three disease agents in nature depends on the intricate relationship between all members of the disease complex. Single viruliferous aphids do not always transmit all three virus agents of the disease and separation of the agents occurs over time and space (Naidu *et al.*, 1999a). Exploratory probes by the aphids into the epidermal and mesophyll cells of groundnut plants could result in the inoculation of GRV and satRNA but these infections would not form sources for further spread because the absence of GRAV prevents virus acquisition by the vector (Naidu *et al.*, 1999b, also see Chapter 1, Figure 1.10). In contrast, single infections with GRAV are symptomless and disease assessment in the field is solely based on the characteristic symptoms, which is only indicative for GRV +satRNA infection.

Improved diagnostic methods include reverse transcription polymerase chain reaction (RT-PCR) to detect each of the three virus agents in plant and aphid tissues (Naidu *et al.*, 1998). The method was applied to investigate the effect of aphid resistance in selected varieties on the transmission of the virus agents of groundnut rosette disease.

4.2. Materials and Methods

4.2.1. Virus source

Aphid and virus susceptible groundnut plants (var. Malimba) containing all three virus agents (GRAV, GRV and satRNA) were continuously available by regularly transferring aphids from these source plants to new young and healthy plants (10 days after planting, DAP). At least 10 aphids were transferred to each plant to maximise transmission of all three virus agents. Acquisition access period (AAP) and inoculation access periods (IAP) were 72 hours. After the IAP, the plants were sprayed with an insecticide (Polysect Insecticide) to kill the aphids and the individual plants were covered in crisp bags (Cryovac Europe, St Neots, UK). Two weeks later, the plants were screened for symptoms which were indicative for GRV + satRNA, while GRAV was assessed by reverse transcription polymerase chain reaction (RT-PCR). Aphid-transmission of both types of rosette, chlorotic and green rosette, was initially conducted but for further evaluation, only the dominant form of the disease in Uganda and Malawi, i.e. chlorotic rosette, was chosen.

4.2.2. Virus detection in leaf and aphid samples

The three agents of GRD in groundnut leaves and aphid samples were detected by RT-PCR (Robertson *et al.*, 1991). RNA was extracted following the protocol for a commercial kit (RNeasy ® Plant Mini Kit, Qiagen, Cat. No. 74904). Primers for specific amplification of nucleic acid sequences from each of the three agents of rosette disease are detailed in Table 4.1 and acquired from SCRI (Taliansky *et al.*, 1996; Deom *et al.*, 2000).

A one step RT-PCR protocol was applied for amplification of the disease agents. The products were acquired from Invitrogen Ltd (Cat.No. 10928-042) and the reaction mix for each sample was as follows:

Reaction buffer x2	12.5µl
Total RNA	2.5µl
Upstream primer	0.5µl
Downstream primer	0.5µl
RT/Taq mix	0.5µl
RNAse-free water	8.5µl

Total	25µl

A product of 597 basepairs (bp) was amplified with primers GRAV-1; GRAV-2. A product of 863 bp was amplified with GRV-1 and GRV-2 primers and satRNA-1 and satRNA-2 primers (Figure 4.1) (Naidu *et al.*, 1998). The RT-PCR was run according to the times and temperatures presented in Table 4.2:

Table 4 1: Primers for the amplification of various regions of causal agents of groundnut rosette disease (Taliansky *et al.*, 1996; Deom *et al.*, 2000).

RT-PCR Primer pairs	Sequence	Specific to
GRAV5[a]	5'-ATGAATACGGTCGTGGTTAGG-3'	GRAV-CP
GRAV3[b]	5'-TTTGGGGTTTTGGACTTGGC-3'	GRAV-CP
S3[a,c]	5'-GGAAGCCGGCGAAAGCTACC-3'	GRV ORF3P& 4P
C3[b,c]	5'-GGCACCCAGTGAGGCTCGCC-3'	GRV ORF3P & 4P
SAT51[a]	5'-GGTTTCAATAGGAGAGTTGC-3'	SatRNA
SAT31[b]	5'-AAATGCCTAGTTTGGGCGTG-3'	SatRNA

[a] Sense-strand primer
[b] Complementary strand primer
[c] S3 and C3, used to obtain *Groundnut rosette virus* open reading frames 3 and 4 (Taliansky *et al.*, 1996)

Table 4 2: Reverse transcription polymerase chain reaction for viral agents of groundnut rosette disease.

RT-PCR process	Temperature (°C)	Time (min)
1. Reverse Transcription	50	2
2. Denaturation of DNA strands	94	2
	94	1
3. Amplification (35 cycles)	55	1
	72	2
4. Extension	72	10

Because GRV and satRNA have always been linked together in nature, only the GRV primers were used for the detection of these agents in the RNA extracts of aphids while in plants, symptom expression was used as indication of GRV + satRNA infection. PCR samples were run on a 1.2% (w/v) agarose gel and visualised with a UV-transilluminator and photographed with a Polaroid MP-4 camera (Figure 4.1).

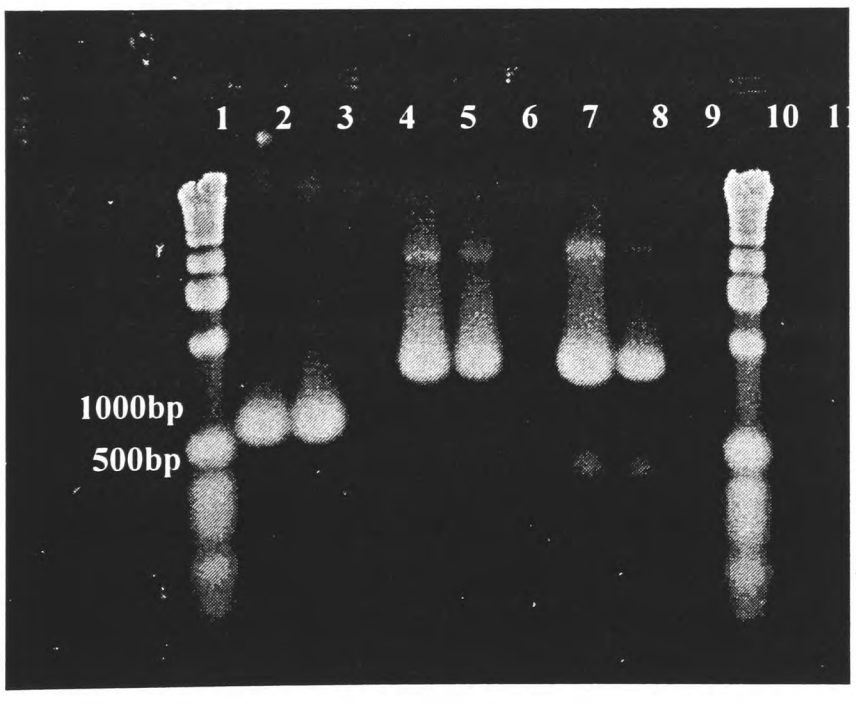


Figure 4 1: Amplification products for the different disease agents: GRAV, GRV and satRNA separated on a 1.2% (w/v) agarose gel and photographed with a polaroid MP-4 camera under UV-light. Lane 1 & 11 = 1 Kb Marker (GibcoBRL). 2-3 = GRAV; 5-6 = GRV; 8-9 = satRNA; 4,7,10 = negative controls (water). Products were amplified using specific primers (Taliansky *et al.*, 1996; Deom *et al.*, 2000).

4.2.3. Transmission of the causal agents of groundnut rosette disease by *A. craccivora*

4.2.3.1. Single aphids and transmission of chlorotic rosette and green rosette

Adult aphids were given an acquisition access period (AAP) of 72 hours on aphid and virus susceptible plants (var. Malimba), which were positive for all virus agents of either the chlorotic or the green variant of groundnut rosette disease (source plants),as tested by RT-PCR prior to the introduction of aphids on the plants. Single aphids from the source plants were transferred to seedlings of JL24 and ICG12991 for a 72h aphid inoculation access period (IAP). Then, the aphids were collected and individually tested for acquisition of GRAV and GRV + satRNA by RT-PCR. The test plants were assessed for the virus agents approximately 14 days after inoculation (DAI). Symptom development indicated infection with GRV + satRNA while RT-PCR was used to assess GRAV infection. Plants that were infected with all three agents or with GRAV only were used as source plants for secondary acquisition (AAP: 48h) by one new adult aphid per plant. The individual aphids were then transferred to new seedlings of the same variety (IAP: 72h). The aphids were again collected after 72h IAP and individually assessed for GRAV, GRV +satRNA by RT-PCR. Plants were assessed for the disease agents 14 DAI.

A randomised block design of 5 plants per variety was applied and replicated 3 times for chlorotic rosette. One plant of JL24 and one of ICG12991 were discarded due to abnormal growing. The data were combined so that 14 replicates for the transmission of GRD virus agents by single aphids on each variety were compared.

The experiment was replicated twice for the transmission of the green variant of groundnut rosette (n= 2x5). The replicates were conducted simultaneously with the experiment on the transmission of chlorotic variant of rosette under identical growing conditions.

4.2.3.2. Transmission of chlorotic rosette virus agents by 5 viruliferous aphids from infected varieties to the aphid and virus susceptible variety CG7

Five viruliferous aphids from the source plants were transferred to each plant of JL24 (susceptible control), ICG SM90704 (GRV + satRNA resistant), ICG12991 (aphid resistant) and ICG SM99540 (aphid resistant). An IAP of 48h rather than 72h was chosen to ensure recollection of the aphids especially from the aphid resistant varieties ICG12991 and ICG SM99540. Groundnut variety ICG SM90704 was selected because of its known GRV +satRNA resistance whereas ICG SM99540 was selected because of its identified aphid resistance (Chapter 3). At 14 DAI, plants were screened for the virus agents. Virus-infected plants with GRAV only or with all three agents, were used as source plants for virus acquisition by 5 non-viruliferous aphids per plant (AAP: 48h). Five aphids were then used for secondary transmission to a new young aphid and virus susceptible host plant, var. CG7 (IAP: 72h). At 14 DAI, the plants of CG7 were screened for the virus agents. A randomised block design was applied and repeated 3 times. Due to bad seed germination of ICG SM99540, the total number of replicates was 12. Also one replicate was discarded from each of JL24 and ICG12991 (n=14) while all replicates of ICG SM90704 were included (n=15).

4.2.4. Aphid performance on virus-infected groundnut plants

Ten viruliferous aphids per plant per variety (JL24; n=8 and ICG12991; n=16) were given an IAP of 72h to maximise inoculation of the virus agents in the seedlings of ICG12991. As a control, plants of both varieties were infested with 10 non-viruliferous aphids. RT-PCR was used to detect GRAV infections and symptom expression was indicative for GRV + satRNA infection in the plants.

The intrinsic rate of increase (Rm) of individual aphids on both varieties containing at least one virus agent was assessed based on the methodology described in Chapter 2. Plants were approximately 25 days after planting (DAP). One adult apterous aphid per plant was allowed to reproduce for 24h on infected and non-infected plants (control) of both varieties. Additionally third generation adult apterous aphids on plants of

ICG12991 only (diseased and control) were also collected and weighed (n=25) on a Mettler AT201 balance (0.01mg).

4.3. Results

4.3.1. Transmission of the causal agents of groundnut rosette disease by *A. craccivora*

4.3.1.1. Single aphids and transmission of chlorotic rosette and green rosette

Aphids acquired all three virus agents from an aphid and virus susceptible source plant (var. Malimba), within a 72h acquisition access period and are referred to as viruliferous, (chlorotic rosette, 89% (n=28); green rosette, 95% (n=20)).

Chlorotic rosette

Almost all viruliferous aphids transmitted at least one of the virus agents to JL24, which was known to be aphid- and virus-susceptible (Figure 4.2). The plants were either totally infected (6/14) or only infected with GRAV (4/14). Totally infected plants of JL24 were also good sources for subsequent virus acquisition by single aphids (5/6) but only one viruliferous aphid transmitted all virus agents successfully to a new test plant and one aphid transmitted only GRAV. Aphids also acquired GRAV from JL24, which were only infected with this agent following a first transmission (2/4), and transmitted it to new plants (2/2).

In contrast to JL24, not one viruliferous aphid transmitted all three virus agents to ICG12991 (Figure 4.3). The majority of aphids only transmitted GRAV to this variety (7/11) and not a single plant was infected with GRV + satRNA. On GRAV-infected plants of ICG12991, only one aphid acquired GRAV but failed to transmit this agent to a new test plant of ICG12991.

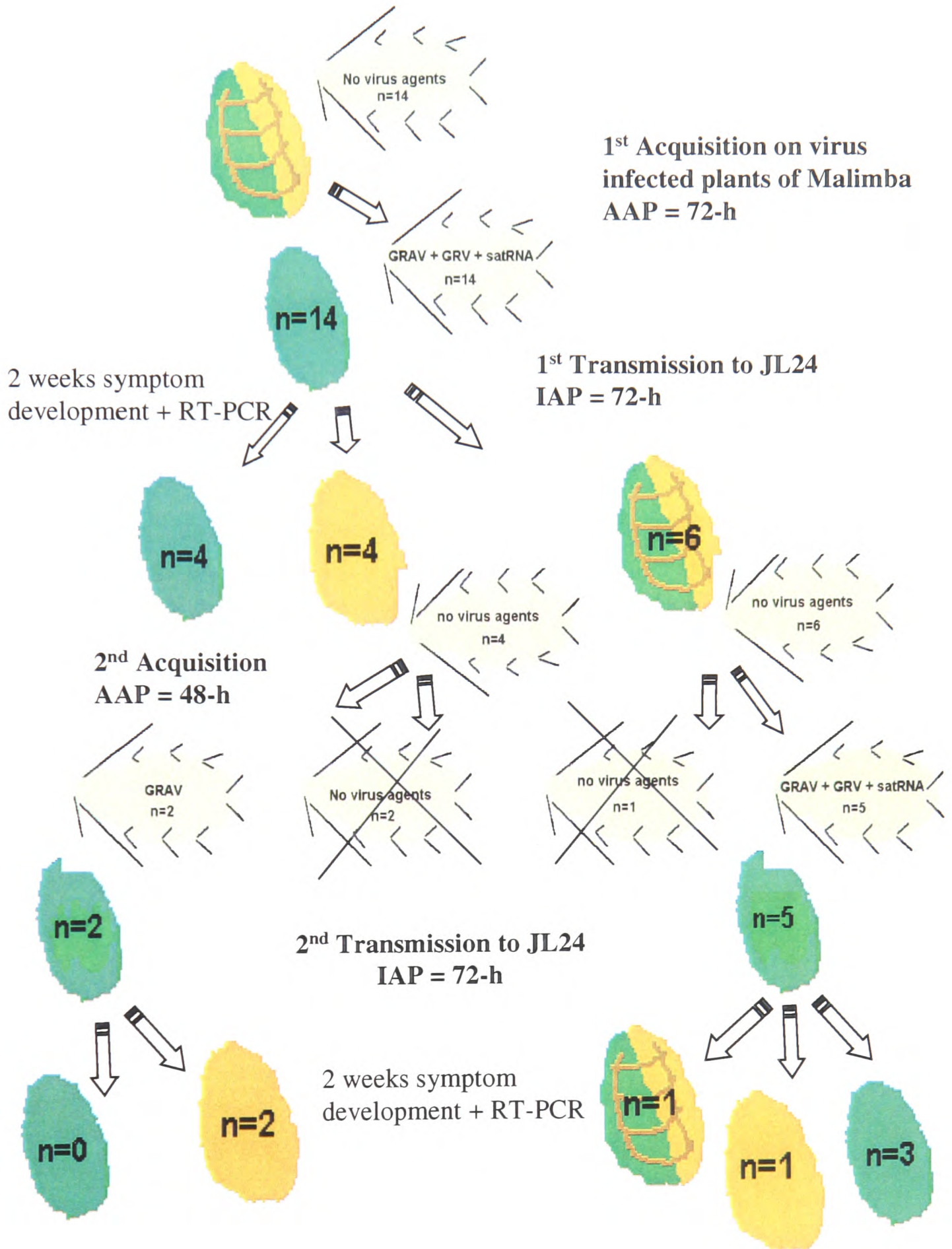


Figure 4 2: Transmission of the chlorotic variant of the groundnut rosette virus agents by single aphids from virus-infected source plants to aphid susceptible groundnut variety JL24 (1st transmission). Plants infected with GRAV or all three agents were used for second transmission tests by single aphids per plant; n= number of replicates.

Healthy plant GRAV-infected plant GRAV+GRV+satRNA- infected plant Vector

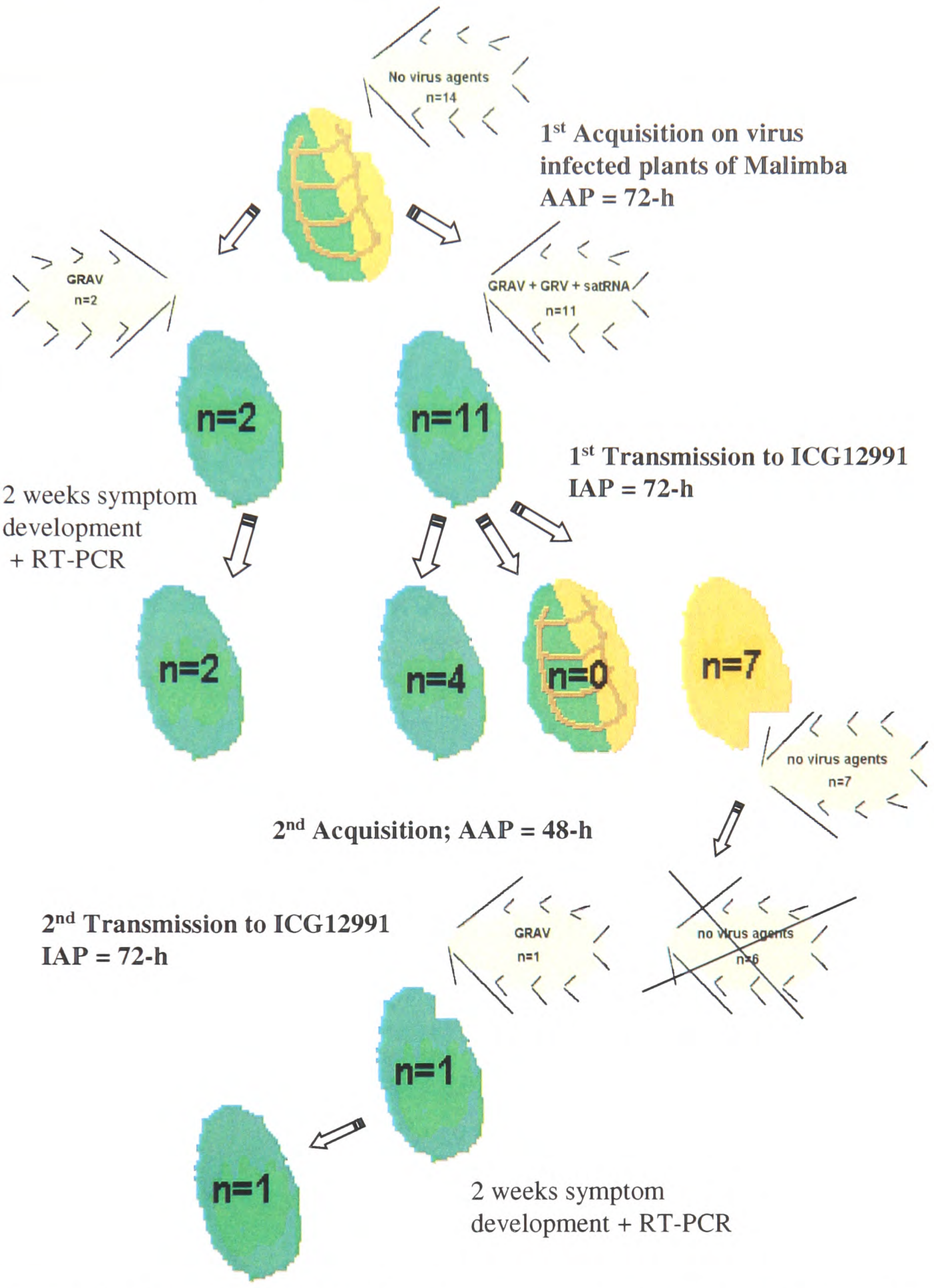


Figure 4 3:. Transmission of the chlorotic variant of the groundnut rosette virus agents by single aphids from virus-infected source plants to aphid susceptible groundnut variety ICG 12991 (1st transmission). Plants infected with GRAV or all three agents were used for second transmission tests by single aphids per plant; n= number of replicates Healthy plant GRAV-infected plant GRAV+GRV+satRNA-infected plant Vector

Green rosette

Single viruliferous aphids transmitted all three virus agents to test plants of JL24 very efficiently (8/10) (Figure 4.4). Subsequent acquisition by aphids from those plants was 100% and transmission resulted in 62.5% of totally infected plants (5/8).

Similar data were obtained for transmission rate to ICG12991 by single viruliferous aphids as those described for the transmission of chlorotic rosette. Most plants were only infected with GRAV (4/7), while the remainder of plants was virus-free (Figure 4.5). Aphids that were only infected with GRAV (2/10), transmitted this agent to a new plant once and the single aphid only infected with GRV + satRNA did not transmit this agent. Not a single aphid was able to acquire GRAV from ICG12991 infected plants and therefore subsequent transmission resulted in virus free plants of ICG12991.

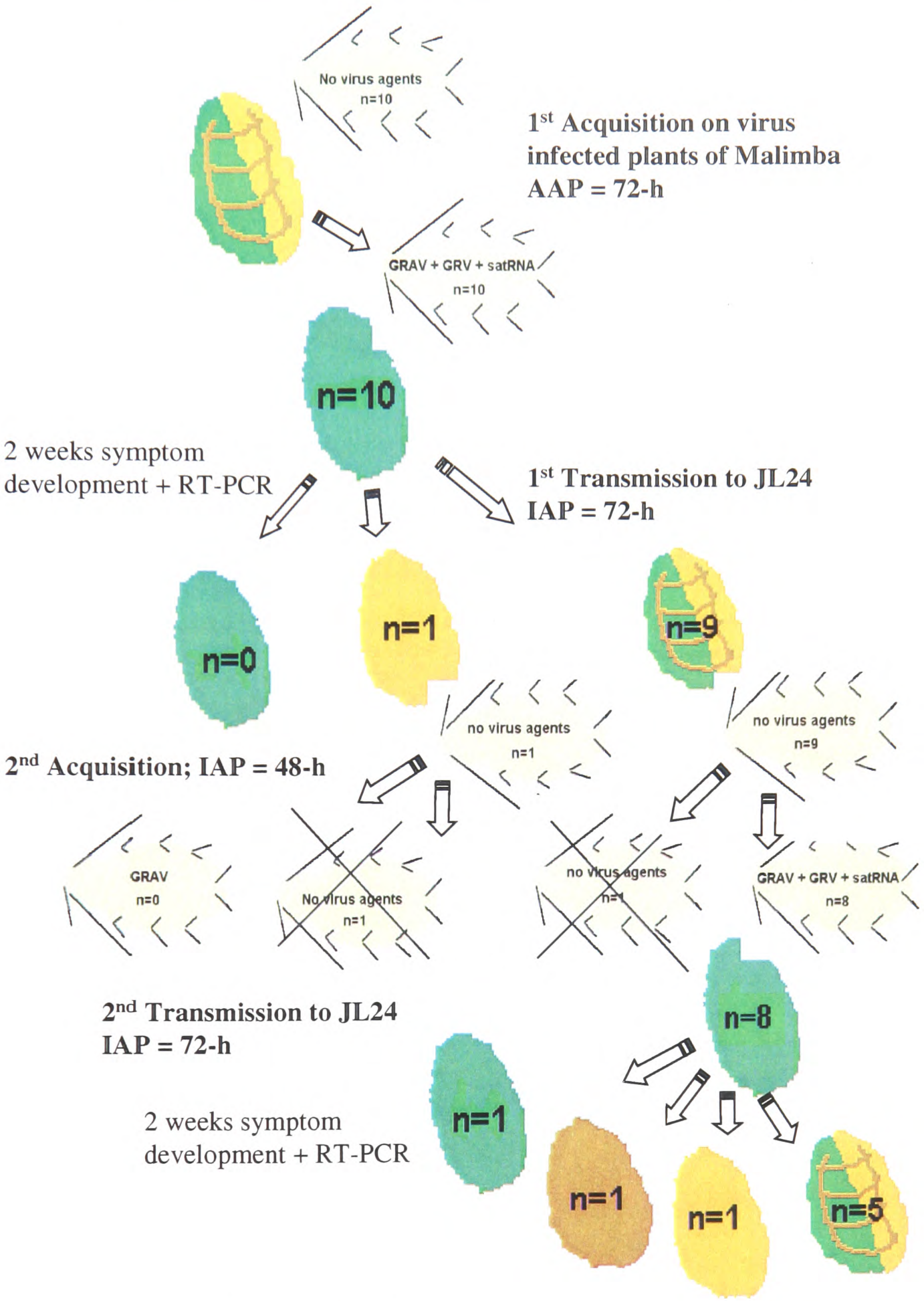


Figure 4 4: Transmission of the green variant of the groundnut rosette virus agents by single aphids from virus-infected source plants to aphid susceptible groundnut variety JL24 (1st transmission). Plants infected with GRAV or all three agents were used for second transmission tests by single aphids per plant; n= number of replicates

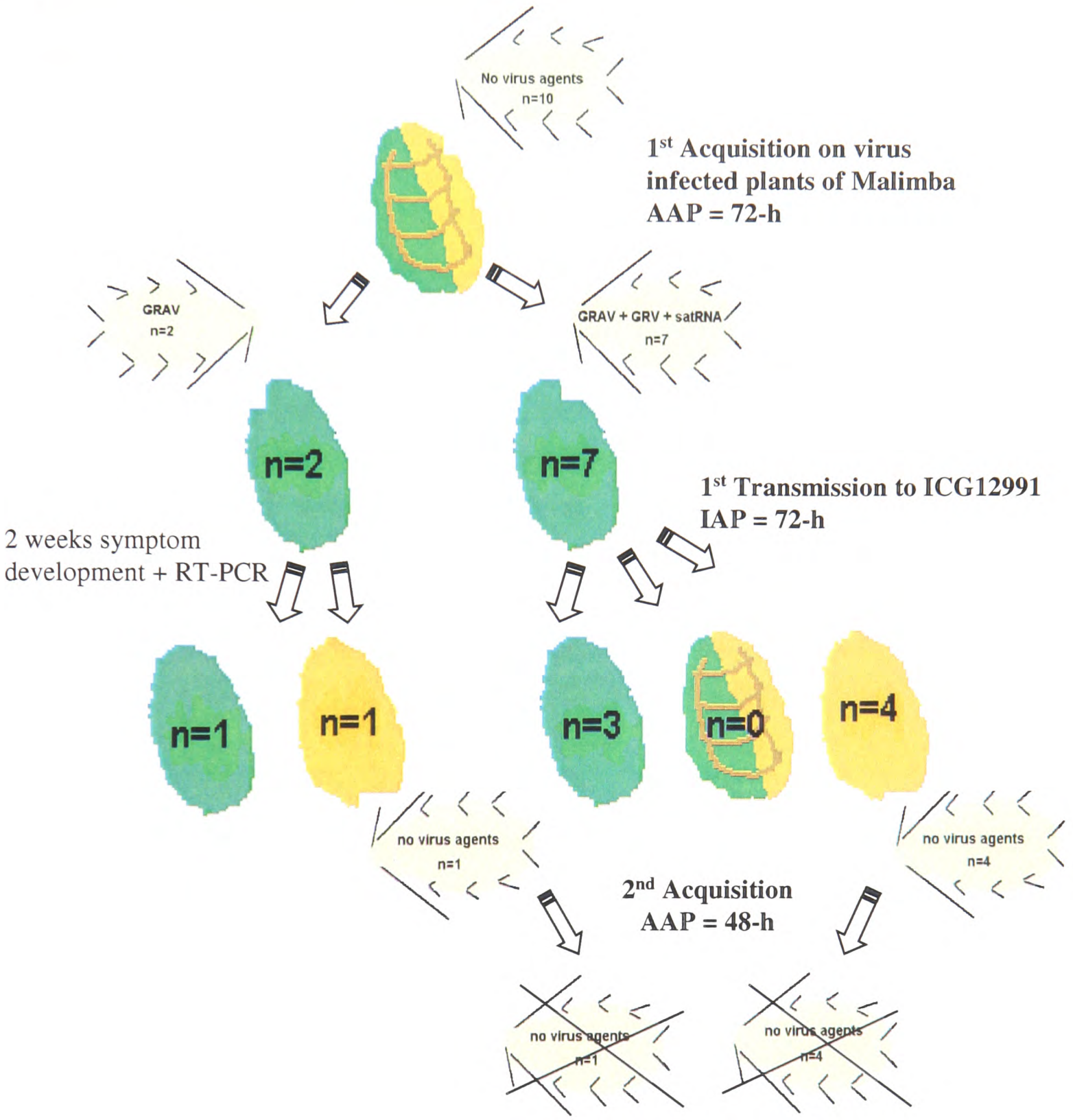


Figure 4 5: Transmission of the green variant of the groundnut rosette virus agents by single aphids from virus-infected source plants to aphid susceptible groundnut variety JL24 (1st transmission). Plants infected with GRAV or all three agents were used for second transmission tests by single aphids per plant; n= number of replicates.

Healthy plant GRAV-infected plant GRAV+GRV+satRNA- infected plant Vector

4.3.1.2. Transmission of chlorotic rosette virus agents by 5 viruliferous aphids from infected varieties to the aphid- and virus-susceptible variety CG7

It was observed that a 72h AAP period would result in a high proportion of viruliferous aphids (>85%; 4.3.1.1.) and therefore the insects were not tested for the virus agents. Results of the transmission experiments to various groundnut varieties are presented in Figure 4.6 a-d.

JL24

JL24 is an aphid- and virus-susceptible variety on which aphids can acquire and transmit the virus agents efficiently (Figure 4.6a). Every single plant of JL24 was infected with GRAV following inoculation of 5 viruliferous aphids and 86% of the plants were totally infected (12/14). From the totally infected plants, 5 non-viruliferous aphids successfully acquired and transmitted all virus agents to CG7 (10/12). The aphids also transmitted GRAV from plants which were only infected with GRAV to CG7.

ICG12991

Transmission of all three virus agents to ICG12991 was successful in only 21% (3/14) following inoculation by 5 viruliferous aphids and the majority of plants (7/14) were only infected with one virus agent, i.e. GRAV (Figure 4.4b). Aphids could not transmit all virus agents to CG7 from totally infected plants of ICG12991 and only GRAV was successfully transmitted to CG7.

ICG SM90704

All plants of this variety were infected with GRAV when 5 viruliferous aphids fed on the plants for 48h and 80% of the plants were infected with all three agents (Figure 4.4c), which was similar to the results for JL24 (Figure 4.6a). Although not all the plants were showing symptoms indicative for GRV +satRNA infection on ICG SM90704, RT-PCR indicated an infection of this virus agent (Figure 4.7). Subsequent transmission from totally infected plants of ICG SM90704 to CG7 however was significantly less (41.5%) when compared to the transmission rate from JL24 (83%). This was mainly due to the low transmission success of GRV + satRNA to CG7 (5/12)

and the majority of CG7 was infected with GRAV only (12/15). Transmission of GRAV to CG7 also occurred from GRAV infected plants only (2/3).

ICG SM99540

As observed for ICG12991, transmission of all three virus agents to ICG SM99540 by 5 viruliferous aphids was not very efficient and half the number of plants remained totally free of virus (6/12). (Figure 4.6d). Disease transmission to ICG SM99540 was only 25% (3/12) and another 25% was only infected with GRAV. Infected plants were not a good source for further virus transmission by aphids and only GRAV was successfully transmitted to CG7.

GRV + satRNA were never successfully transmitted from virus infected plants of the resistant varieties ICG12991 and ICG SM99540 to the susceptible variety CG7.

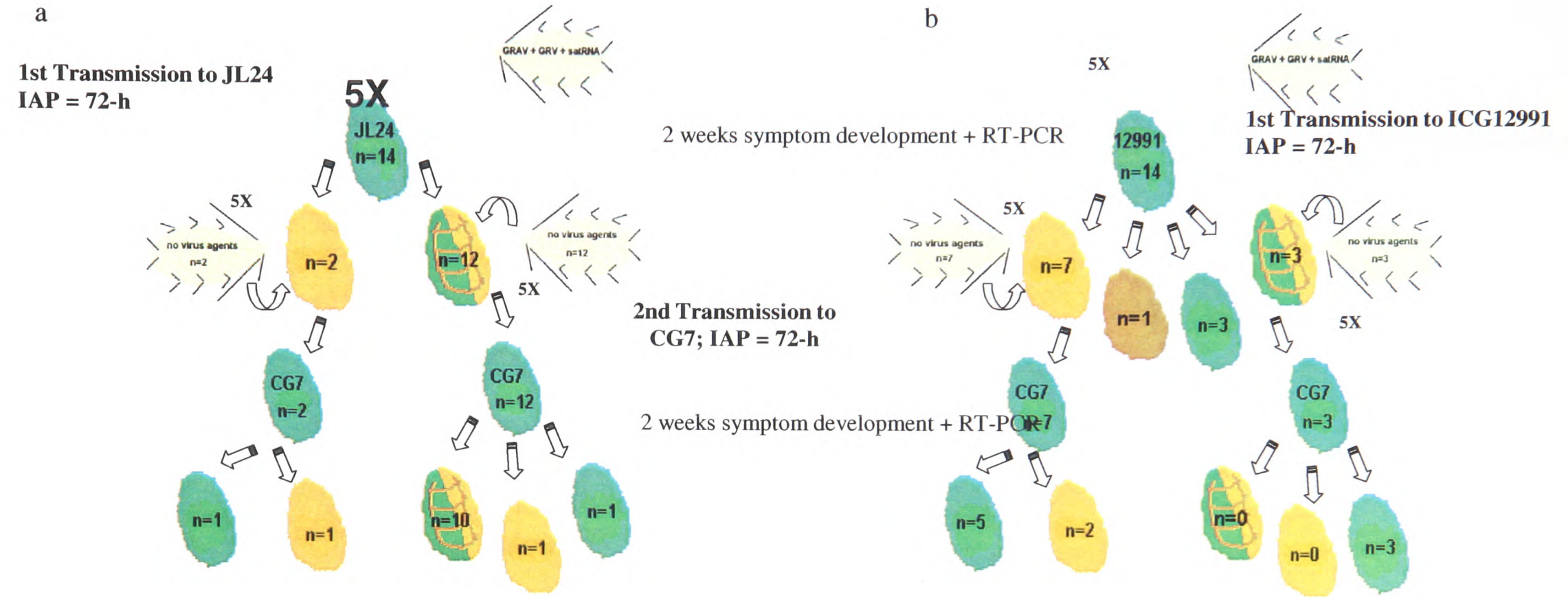


Figure 4 6a-b: Transmission of the chlorotic variant of the groundnut rosette virus agents by 5 viruliferous aphids from virus-infected source plants to aphid susceptible JL24 (a) and resistant groundnut variety ICG12991 (b) (1st Transmission). Plants infected with all three agents or GRAV only following first transmission were used as source plants for 5 non-viruliferous aphids to acquire and transmit virus agents to the susceptible variety CG7 (2nd transmission)

Healthy plant GRAV-infected plant GRAV+GRV+satRNA- infected plant GRV+satRNA-infected plant Vector

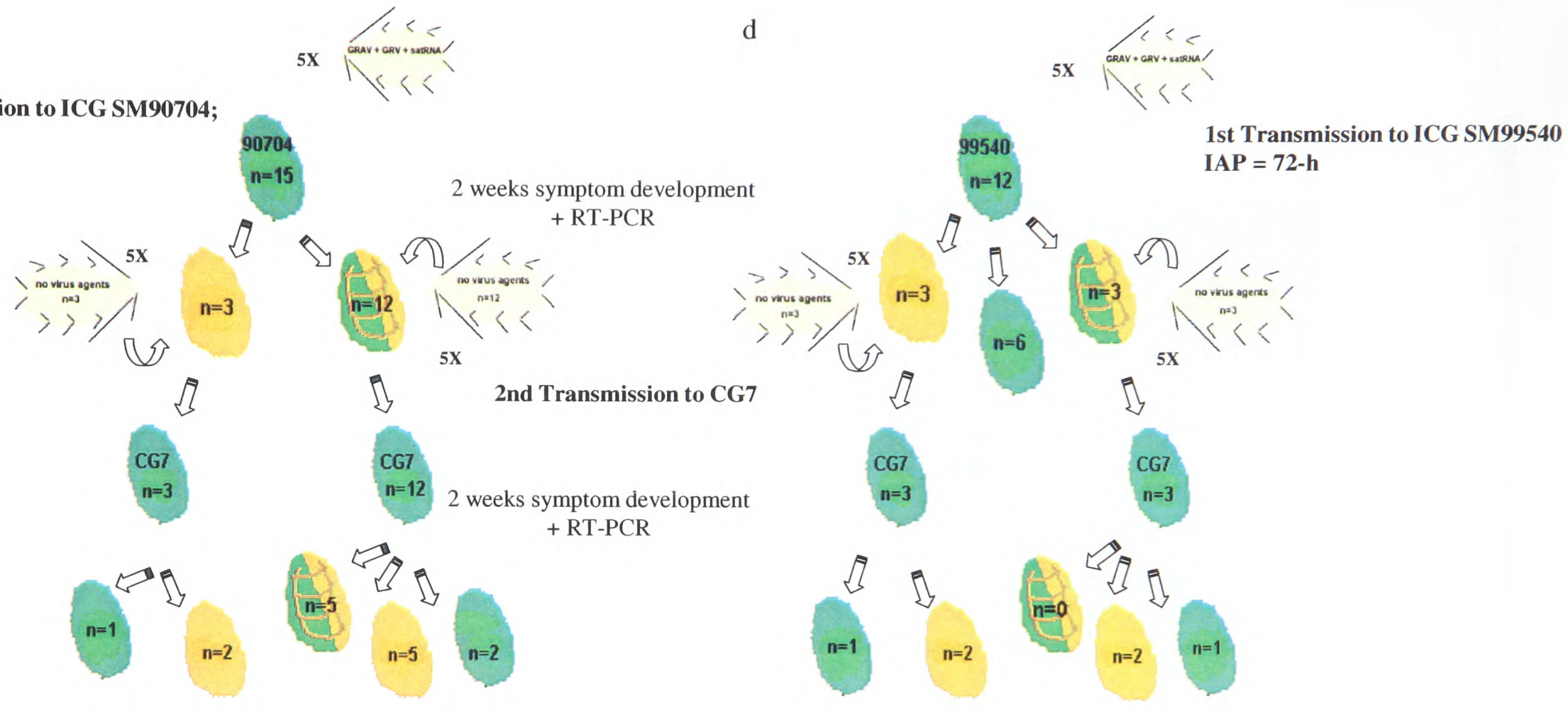


Figure 4.6c-d: Transmission of the chlorotic variant of the groundnut rosette virus agents by 5 viruliferous aphids from virus-infected source plants to aphid susceptible but GRV resistant variety ICG SM90704 (c) and the aphid resistant groundnut variety ICG SM99540 (d) (1st Transmission). Plants infected with all three agents or GRAV only following first transmission were used as source plants for 5 non-viruliferous aphids to acquire and transmit virus agents to the susceptible variety CG7 (2nd transmission)

Healthy plant GRAV-infected plant GRAV+GRV+satRNA- infected plant Vector

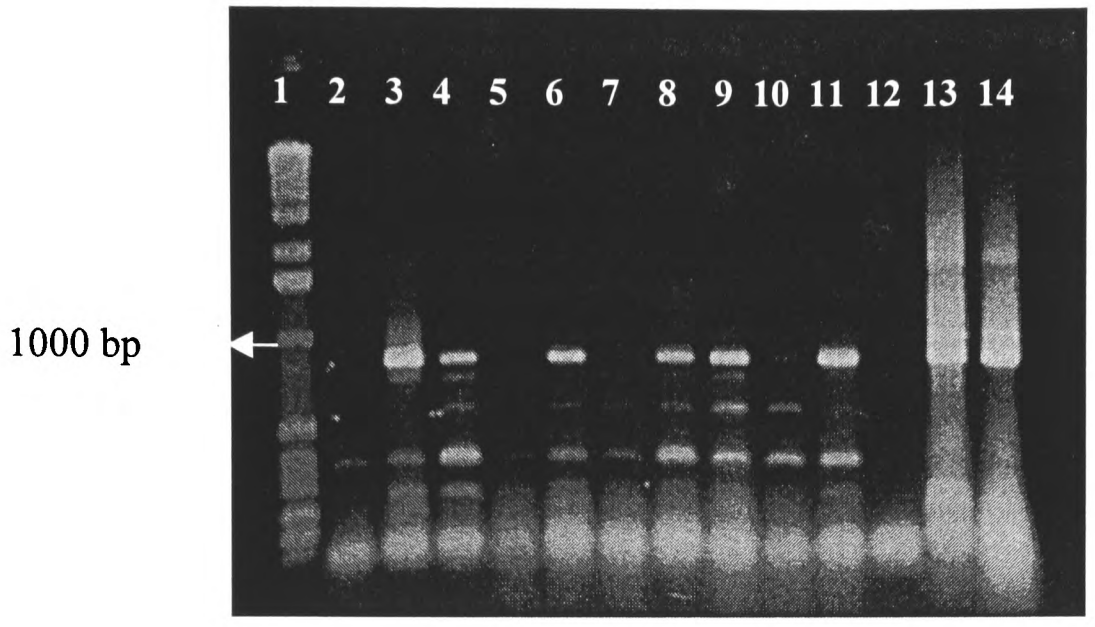


Figure 4 7: Amplification of GRV (863 bp) from ICG SM90704 following inoculation of 5 viruliferous aphids per plant (7 DAP). Lane 1 = 1Kb Marker (GibcoBRL); lane 2-11 = ICG SM90704 samples; lane 12 = water control; lane 13 = positive control; lane 14 = PCR control.

4.3.2. Aphid performance on virus-infected groundnut plants

A high number of viruliferous aphids per plant (>10 aphids per plant) was insufficient to obtain totally virus-infected plants of ICG12991. Only 12.5% (2/16) of the plants was infected with all three virus agents compared to a 100% infection rate on JL24 (Table 4.3). All virus-infected plants of both varieties were used to measure parameters of aphid performance.

The intrinsic rate of population increase (Rm) was significantly increased on infected plants of both ICG12991 and JL24 compared to the Rm on healthy plants ($P<0.01$; Mann Whitney *U* test) because aphid fecundity was significantly higher on infected plants than on healthy plants (Table 4.4a,b). Only 50% of the aphids survived on virus-free plants of ICG12991, while all aphids survived on the infected plants (Table 4.4b). Adult aphids from the third generation weighed significantly more on infected plants of ICG12991 than those on healthy plants ($P<0.0001$; Student t-test, n=25). The mean weight (± S.E.) of the insects on infected plants was 0.63mg (±0.03) compared to 0.40 (±0.02) on virus-free plants.

Table 4 3: Infection (%) of GRD agents on JL24 and ICG12991 (7 DAP) by 10 viruliferous aphids/plant. AAP was 72h on source plants, IAP of 48h on test plants. n= number of replicates.

Transmission of GRD agents by 10 viruliferous aphids per plant	**Varieties**	
	JL24 (n=8)	**ICG12991 (n=16)**
All three agents	8	2
GRAV	0	2
GRV + satRNA	0	3
No agents	0	9

Table 4 4a: Aphid performance on healthy and virus-infected plants (21DAP) of groundnut variety ICG12991. D= aphid development time in days, Md= number of offspring in D days, and Rm =intrinsic rate of increase. n= number of replicates for each measurement, M= aphid mortality.

Virus-infected plants of ICG12991 (n=7)	**Virus agents**		**Aphid performance**			**Virus free plants of ICG12991 (n=11)**	**Aphid performance**		
	GRAV	**GRV + satRNA**	**D (n=7)**	**Md (n=7)**	**Rm (n=7)**		**D (n=9)**	**Md (n=6)**	**Rm (n=6)**
1	+	+	6	63	0.51	1	7	23	0.33
2	+	+	6	63	0.51	2	7	28	0.35
3	+	-	6	33	0.43	3	6	25	0.40
4	+	-	6	52	0.49	4	7	M	M
5	-	+	6	60	0.50	5	7	M	M
6	-	+	6	56	0.50	6	6	16	0.34
7	-	+	7	63	0.44	7	6	28	0.41
						8	M	M	M
						9	6	15	0.33
						10	6	M	M
						11	M	M	M
Median			**6**	**60**	**0.50**	**Median**	**6**	**24**	**0.34**

Table 4.4b: Aphid performance on healthy and virus-infected plants of groundnut variety JL24 (21DAP). D= aphid development time in days, Md= number of offspring in D days, and Rm =intrinsic rate of increase. n= number of replicates for each measurement.

Virus-infected plants of JL24 (n=8)	**Virus agents**		**Aphid performance**			**Virus-free plants of JL24 (n=8)**	**Aphid performance**		
	GRAV	**GRV+ satRNA**	**D (n=8)**	**Md (n=8)**	**Rm (n=8)**		**D (n=8)**	**Md (n=8)**	**Rm (n=8)**
1	+	+	6	81	0.54	1	6	74	0.53
2	+	+	6	73	0.53	2	6	71	0.53
3	+	+	6	84	0.55	3	6	76	0.53
4	+	+	6	73	0.53	4	6	66	0.52
5	+	+	6	70	0.52	5	6	65	0.52
6	+	+	6	76	0.53	6	6	68	0.52
7	+	+	6	76	0.53	7	6	66	0.52
8	+	+	6	73	0.53	8	6	72	0.52
Median			**6**	**75**	**0.53**	**Median**	**6**	**70**	**0.52**

4.4. Discussion

Efforts to control virus diseases in agricultural crops have largely focused on planting virus-free material, minimising virus infection entering the crop and/or the spreading within it, including the use of insecticides to control the insect vector (Jones, 1987). Insecticide applications to control insect vectors, and indirectly the viruses that they spread, have been successful to control semi-persistent and persistent viruses (Perring *et al.*, 1999) but deployment of insecticides is now increasingly questioned especially in developing countries where highly hazardous practices using inappropriate and unapproved products are widespread (Williamson, 2003).

To control groundnut rosette disease, breeding programmes in sub-Saharan Africa have focused on the identification of **virus-resistant varieties** but the results presented here show that **vector-resistant varieties** also have great potential to control this disease. Aphid resistance in ICG12991 was illustrated in Chapters 2-3 and the variety was totally susceptible to the virus agents of rosette disease (Merwe van der *et al.*, 2001; Figure 1.2, Chapter 1). To investigate the effect of vector resistance on the transmission of the virus agents, detection methods to ascertain infection in the vector and plant tissues are required. It was already found that a single viruliferous aphid can transmit the three virus agents separately in time and space in different proportions and that more plants are infected with all three agents when more than one viruliferous aphid per plant is used in transmission tests (Murant, 1990; Naidu *et al.*, 1999a). The results presented here supported this spatio-temporal separation of the virus agents and some plants were infected with GRAV only, while others were infected with GRV and satRNA only or with all three agents. Compared to the susceptible variety JL24, not one plant of ICG12991 was infected with all three agents using single viruliferous aphids per plant. Approximately half the plants of ICG12991 was infected with GRAV only and this result was consistent for the transmission of the chlorotic and green variant of rosette disease using single viruliferous aphids. Increasing the number of viruliferous aphids to five per plant or even 10 per plant only slightly increased infection of all virus agents on ICG12991 and most plants were infected with GRAV only. In contrast, almost all the plants of JL24 were infected with all three agents under this high aphid and virus pressure.

The plants were 7 days after planting at the time of the transmission experiments and previous results already showed that infection decreased when plants were older (Naidu *et al.*, unpublished data). This is most likely correlated with the aphid preference for younger plants (Farrell, 1976a). Nonetheless, even under very high pressure of viruliferous aphids, virus infection on young plants of ICG12991 was very low. These results are very important in terms of virus infection in the field because primary infection of the virus must be introduced into the crop by viruliferous aphids before secondary spread to neighbouring plants can occur. Additionally, non-viruliferous aphids were strongly inhibited to acquire virus particles from infected plants of ICG12991 and transmit the disease to the aphid and virus susceptible variety CG7. Therefore, apart from primary infection, secondary spread of the disease in the field from virus-infected plants of ICG12991 would be greatly inhibited. The observations presented here could explain the low disease incidence reported repeatedly for ICG12991 (Merwe van der & Subrahmanyam, 1997, Naidu *et al.*, 1999b; Subrahmanyam *et al.*, 2000). These reports however only rely on symptom expression, which indicate infection of GRV + satRNA but lack information on GRAV infection. It was now illustrated that ICG12991 was more readily infected with only GRAV, at least when plants were very young.

Similar results as described for the transmission experiments on ICG12991 were obtained on ICG SM99540. This variety was also identified as vector resistant (Chapter 3). It was illustrated that resistance to the aphid vector in ICG12991 and ICG SM99540 inhibited virus transmission and acquisition. This type of vector resistance was more effective than resistance to the virus agents. ICG SM90704 was reported as GRV +satRNA resistant but under high disease pressure the resistance was broken down and almost all plants were infected with all three virus agents. Although plants did not always express obvious symptoms, GRV + satRNA infection was transmitted to CG7 in subsequent transmissions and detected with RT-PCR. There are also reports of partially expressed symptoms on this variety (P.J.A. van der Merwe pers. comm.) and more research is required to better understand the GRV + satRNA- resistance in ICG SM90704. It has now been hypothesised that the resistance mechanism may be based on the restriction of the replication of GRV + satRNA. Therefore symptoms may not

always be expressed and screening trials that included ICG SM90704 probably underestimated GRD-incidence in this variety. Low titres of GRV +satRNA would additionally decrease encapsidation rates of this agent into the coat protein of GRAV, and therefore lower the probability for acquisition and transmission by aphid vectors. This may be supported by a delayed expression of symptoms on graft- and aphid inoculated plants of ICG SM90704 compared to expression on graft- and virus inoculated plants of susceptible varieties such as JL24, CG7 and ICG12991 (Naidu *et al.*, unpublished data). Quantification techniques would allow testing this hypothesis. Host plant resistance associated with a decrease in virus accumulation, coupled with decreased symptom expression, has been described for other viruses such as *Tomato yellow leaf curl virus* (TYLCV, Fam. Geminiviridae) (Maruthi *et al.* 2003), *Potato leaf roll virus* (PLRV, Fam. Luteoviridae) (Barker & Harrison, 1985, 1986), *Cucumber mosaic virus* (CMV, Fam. Bromoviridae) (Wood & Barbara, 1971; Maule *et al.*, 1980), *Maize streak virus* (MSV, Fam. Geminiviridae) (Bock, 1982) and *African cassava mosaic virus* (ACMV, Fam. Geminiviridae) (Hahn *et al.*, 1980).

It has been reported that groundnut plants showing symptoms of rosette disease were more attractive to aphids, aphid populations developed faster, and higher number of winged adults were produced than on healthy plants (Réal, 1955; F.M. Kimmins, unpublished data). There is sufficient evidence in the literature which showed that the biochemical changes in virus-infected plants can affect vector fecundity, longevity and survival to varying degrees, either positively or negatively (Kennedy, 1951; Baker, 1960; Sohi & Swenson, 1964; Gildow, 1983; Montllor & Gildow, 1986). The interactions between vector and virus are considered adaptive and would favour aphid dispersal and virus spread within and between crops (Kennedy, 1951; Sohi & Swenson, 1964; Gildow, 1983). On groundnut, aphid fecundity was significantly increased on plants of JL24 and ICG12991 infected with one or more of the virus agents of groundnut rosette disease. The higher weight of adult aphids on infected plants of ICG12991 could also represent physiological, biochemical or morphological changes in the plants, beneficial to the aphids' survival rate and overall performance. This has been reported for other aphid species. *Myzus persicae* for example produced more offspring on a resistant variety of potato infected with PLRV than on healthy plants of a

susceptible variety and another aphid vector, *Aphis fabae*, reproduced approximately 1.5 times faster on *Sugar beet mosaic virus* (BtMV, Fam. *Potyviridae*) infected plants than on healthy plants (Baker, 1960). *Barley yellow dwarf virus* (BYDV, Fam. *Luteoviridae*) and *Brome mosaic virus* (BMV, Fam. *Bromoviridae* induced alatae production in the cereal grain aphids (*Sitobion (=Macrosiphum) avenae* and *Rhophalosiphum padi*, on infected barley and oat plants compared to aphids on healthy plants (Gildow, 1983; Montllor & Gildow, 1986).

It was not opted to further elucidate the correlation between the increased performance of *A. craccivora* and virus-infection in groundnut. There are abundant examples in the literature on this phenomenon in other aphid-plant interactions to illustrate how virus infection can change the plant's physiological, biochemical and morphological characteristics (Harpaz & Applebaum, 1961; Thresh, 1967; Matthews, 1981; Ajayi, 1986; Costa *et al.*, 1991; Sandström *et al.*, 2000). Instead it was decided that further study was needed to understand the mechanism of resistance since this could provide background information on the durability of the vector resistance.

Laboratory and field studies on the resistance in ICG12991 to the groundnut rosette disease complex required an investigation of the underlying mechanism of resistance. The identification of resistance mechanisms is of primary necessity in the process of building new strategies to control groundnut rosette in key area of sub-Saharan Africa.
In the next chapter the feeding behaviour of *A. craccivora* on resistant and susceptible groundnut varieties will be examined.

In Summary:

1. Resistance to the vector to *A. craccivora* in ICG12991 and ICG SM99540 prevented transmission of all three virus agents.
2. Higher inoculation pressure did not lead to higher infection of all virus agents on ICG12991 and ICG SM99540 compared to the virus-resistant ICG SM90704 and the susceptible control JL24.
3. Virus acquisition of all three agents from infected plants of ICG12991 and ICG SM99540 and subsequent transmission was not observed.

4. Virus resistance in ICG SM90704 was broken down under high pressure of viruliferous aphids.
5. Virus acquisition and transmission of all three agents of infected plants of ICG SM90704 was greatly reduced compared with plants of JL 24.
6. GRV +satRNA infection of ICG SM90704 did not always result in obvious symptom expression.
7. Aphids performed significantly better on virus-infected plants of JL24 and ICG12991 than on virus-free plants of either of the varieties.

CHAPTER 5

APHID FEEDING BEHAVIOUR ON GROUNDNUT AND MECHANISM OF RESISTANCE IN VARIETY ICG12991

5.1. Introduction

Several resistance mechanisms have been proposed for various aphid-plant interactions, which range from deterrent volatiles to toxic compounds in the phloem (Nottingham *et al.*, 1991; Dreyer & Campbell, 1984; Weibull *et al.*, 1986). A balance between positive and negative stimuli will influence aphid behaviour at each stage of host plant selection and ultimately the insect may leave the plant (Niemeyer, 1990; Chapter 1, Figure 1.13). Aphids mainly use internal chemical or mechanical plant factors which are encountered during stylet penetration in epidermal, mesophyll and phloem tissues to accept or reject a plant. A mechanical mechanism could involved the toughness of the tissues (Tjallingi, 1990b), whereas a chemically based resistance mechanism would imply a lack of phagostimulants or the presence of phagodeterrents (Wensler & Filshie, 1969; Tjallingii, 1985a,b; Powell, 1991; Tjallingii & Hogen Esch, 1993) or constituents of the apoplastic fluids (Wensler & Filshie, 1969; Urbanska *et al.*, 1994; Sauge *et al.*, 1998). Aphid saliva and the mechanical effects of stylets during penetration of plants may also be involved (Kimmins, 1986, 1988). Aphid saliva is assumed to activate unique defensive responses in plants, but may also counter them (Miles, 1999).

Plant resistance to aphids is often reflected in their feeding behaviour and this can be monitored using the Electronic Penetration Graph technique (EPG) developed by Tjallingii (Tjallingii, 1978a,b, 1988, 1990a,b). This technique gives detailed information on stylet activity as the stylets penetrate the plant and enables distinction between intra- and extracellular stylet tip position in plants, according to the recorded signal potential level (Tjallingii, 1985b). Specific waveforms of the EPG have been correlated with aphid salivation and ingestion from plant tissues (Tjaliingii, 1978a,b,

1994; Kimmins & Tjallingii, 1985), which are important parameters in virus-vector plant interrelationships.

The EPG in combination with other laboratory-based observations was used to study the feeding behaviour of *A. craccivora* on groundnut variety ICG12991. This variety was markedly resistant to the aphid vector under both laboratory and field conditions and therefore reduces transmission of *Groundnut rosette virus* (Chapter 4). Data on the feeding behaviour were needed in this study to better understand the mechanism of resistance. The relationship between aphid feeding behaviour on ICG12991 and other varieties of interest in relation to the transmission of the virus agents of groundnut rosette disease is discussed elsewhere (Chapter 8).

5.2. Materials and Methods

5.2.1. Aphid behaviour on aphid-susceptible and aphid-resistant groundnut varieties under laboratory and glasshouse conditions

The behaviour of individual alatae aphids on 2 groundnut varieties was observed. One individual aphid was placed on the youngest unfolded leaf of the plants. Using a magnifier and stopwatch, the time before aphids made their first probe, the number of probes and the lengths of every probe were recorded over a 15min period. The start of a probe was considered to be when the aphid had been immobilised with the antennae folded to the back for 5 seconds. Eight insects per variety were observed and the individual test-plants were chosen in a randomised manner. The varieties were ICG12991 (aphid-resistant; Chapters 2, 3), and JL24 (aphid susceptible, control). After 24h, the plants were screened for the aphid and recorded 0-1 for absence or presence. The experiment was first conducted at NRI on plants that were 14 days after planting (DAP, 4^{th} leaf) and then repeated under glasshouse conditions at ICRISAT-Malawi with plants 28 DAP (6^{th} leaf).

5.2.2. Honeydew excretion by *A. craccivora* on leaf and flower tissue of aphid-susceptible and aphid-resistant groundnut varieties in the laboratory

One individual adult apterous aphid of a Ugandan population was confined in a clip-cage on the abaxial surface of the youngest developed leaflet. A piece of water- and oil-sensitive paper (TeeJet, Spraying Systems Co®) was placed into the bottom half of the clip-cage. The reaction of the paper with the excretion product of feeding aphids (honeydew) causes a colour change, which allows the measurement of the number and size of the honeydew deposits (HDs). A new piece of indicator paper was applied at regular intervals (2, 4, 6, 8, 24 and 48h) without disturbing the insects. The number and size of HDs excreted by aphids feeding on ICG12991 was compared with those feeding on JL24. The plants were 10 DAP to secure survival of the aphids on ICG12991 (Chapter 2). The number of HDs was counted and the diameter measured using a

binocular microscope (magnification, 10 x 12). HDs excreted by first instar nymphs were chosen randomly and also measured. The experiment was replicated 10 times for each variety.

To assess honeydew production on flower tissue, one adult apterous aphid was placed on the flower stem. Once the aphid was settled on the stem, the plant was placed into position using a laboratory clamp so that the HDs could fall onto the indicator paper placed only a few cm lower. For each variety 30 randomly chosen HD were measured. Eleven replicates of JL24 and 18 on ICG12991 were analysed and compared. Individual flowers exist for approximately 24h in the laboratory so honeydew collection by feeding aphids on flowers was restricted to 24h.

The number and size of honeydew deposits were compared between the two varieties using Student t-tests.

5.2.3. Localised areas of cell damage on ICG12991 and aphid feeding

Localised areas of cell damage were observed on leaf tissue of ICG12991 following aphid infestations, which were completely absent in other varieties including JL24, CG7 and ICG SM90704. They could be identified by small lesions on the adaxial side of the leaves and small areas of collapsed cells on the abaxial side corresponded therewith.

For more detailed observations, Scanning Electron Microscopy (SEM) was applied. Specimens of leaf tissue of ICG12991 were prepared using the 'critical point drying' technique. The method was chosen because the exposure of fresh leaf tissue to a very high vacuum in the electron microscope would cause the cells to dry out and collapse (S. Reardon pers. comm.). The drying process can be divided into three stages:

1. Fixation

The specimens were immersed in a chemical 'fixative' solution overnight. Gluteraldehyde, mixed with a phosphate buffer (0.05M) was used as the fixative to kill and preserve the cells.

2. *Dehydration*

Water was gradually removed from the specimen and replaced with acetone by moving it through a series of the following solutions:

Phosphate buffer (0.05M)	10 min
Phosphate buffer (0.05M)	10 min or storage in fridge
50% Acetone	20 min
70% Acetone	20 min or overnight in fridge
80% Acetone	20 min
90% Acetone	20 min
100% Acetone*	45 min
100% Acetone*	45 min
100% Acetone*	45 min

*Acetone was stored over anhydrous sodium sulphate and molecular sieve to ensure no contamination with water

3. *Critical Point Drying*

The specimens were transferred to the critical point dryer container filled with dry acetone (100%) overnight. The individual specimens were then ready to mount with an adhesive (Araldite) to a metal stub, which acts as the specimen holder in the SEM. The specimen, adhesive and stub were coated with a very thin layer of gold in a 'sputter coater' for 4 min and visualised under the SEM. Twenty areas of localised areas of collapsed cells, induced by aphid probing/feeding on the abaxial leaf tissue of ICG12991 from at least 6 different plants were photographed. The adaxial surface did not show any area of cell collapse and this reaction was also not visible on either side of the leaves of a control variety JL24.

5.2.4. Leaf clearing technique and quantification of areas of cell damage

Adult aphids were placed in a clip-cage for 24h on the youngest undeveloped leaves of groundnut varieties ICG12991, ICG9723 and JL24. ICG9723 was selected as localised areas of necrosis were observed in a range of varieties as a response to aphid infestation when screening for vector resistance (Figure 5.1). Aphids were given 24h access on the youngest expanding leaves of plants, which were approximately 12 days after planting and insect-free plants served as a control. The aphids were removed and the leaves were collected after they were expanded and then immersed in methanol overnight to clear out the chlorophyll. The leaves were then further cleared in a saturated solution of chloral hydrate (3mg/1ml H_20). Localised areas of accumulated compounds, presumably phenolics (Bennett *et al* ., 1999) were visible on the cleared tissues and photographed with a camera-fitted-microscope, magnification (10X12.5). The photographs were modified in two different ways in Paintshop 7.0 prior to analysis by an image analyser (Quantmet 520) to quantify the area affected. For each variety, 50 photographs were analysed. No lesions were observed for JL24 but photographs were taken when irregularities were found.

Modification of photographs: method 1.

The photographs were altered to greyscale in Paintshop 7.0 and then modified using the contrast/brightness function until the area of interest appeared as clear as possible. This method was chosen as the image analysis was based on greyscales and in XIMAGE, grey-detection was increased until background noise started to appear.

Modification of photographs: method 2.

A colour with following characteristics was selected to represent the colour of the hypothesised phenolics in the photographs: Red, 138; Green, 103; Blue, 37; Hue, 27; Saturation, 147 and Light 88. Colours within a certain threshold of this selection were altered to black. The threshold was based on the appearance of background noise.

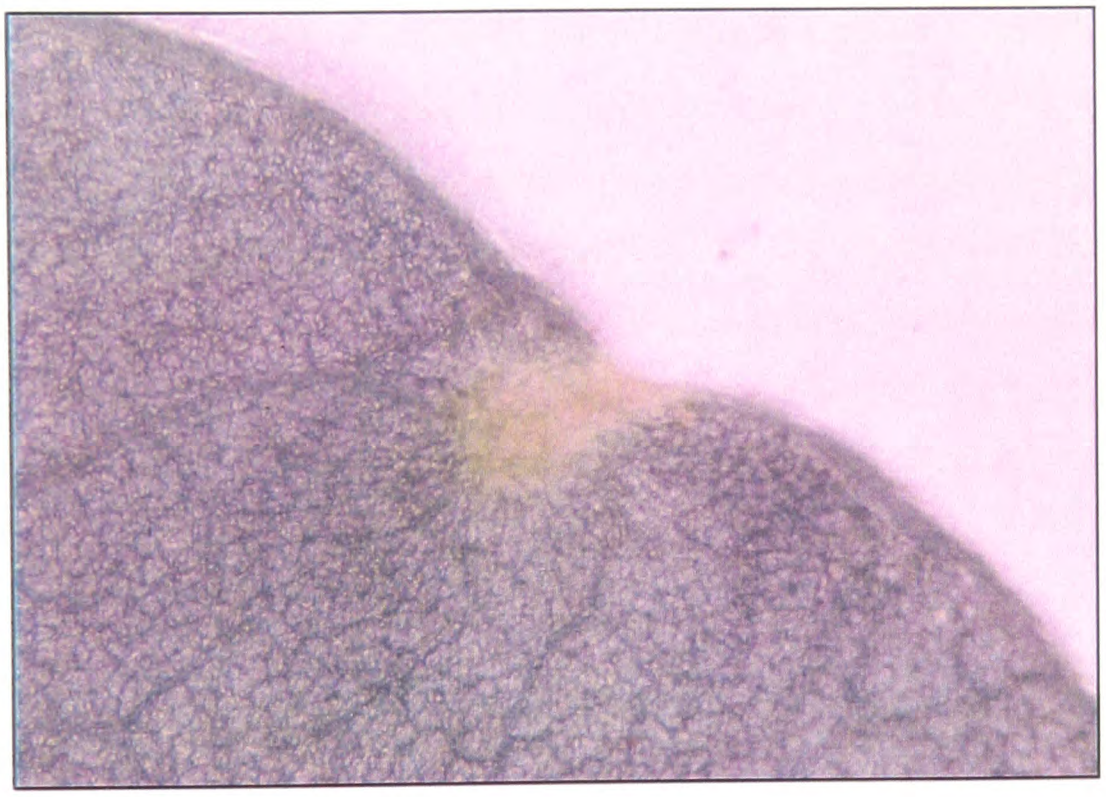


Figure 5 1: Necrotic areas on groundnut leaves following aphid feeding on variety ICG5240. These areas were also found on ICG9723, ICG11735, ICG11788 and ICG11649.

5.2.5. Aphid feeding behaviour on aphid-susceptible and aphid-resistant groundnut varieties and cell damage

The Electronic Penetration Graph (EPG) technique (Tjallingii, 1978a,b) was used to record aphid feeding behaviour. A 2 cm gold wire (diam 20 μm) was attached to the dorsum of the aphid by conductive silver paint and connected to the amplifier (10^9 Ω input resistance). Different conductive silver paints were used (Demetron, ethanol based and water based solution). The aphids did not appear to be affected by the different paints and therefore the results were analysed together. An electrode was placed into the potting soil to connect the supplied voltage (± 100 mV) and the whole set-up was placed in a Faraday-cage. Aphids were deprived of food for approximately 2 hours during the set-up process of 8 recording channels. All signals were recorded on a PC hard disk and analysed by Stylet 3.7 software. Different waveforms that can be distinguished in the EPG and used for analysis are summarised in Figure 5.2 and Table 5.1. The recorded waveforms were according to the work of Tjallingii (1990b) and could be clearly identified.

Experiment 1

Aphid feeding behaviour on two varieties was compared over a 4h recording period. The varieties were JL24 and ICG12991 and 14 DAP (4^{th} leaf stage). Aphids were collected at 10 am for standardisation of the procedure. During the recording period, the position of the aphids on the leaves was recorded on a sheet of paper every 20-30 min. to recover the areas of aphid feeding. The plants were placed into the controlled environment rooms for 24h at the end of the experiment. The leaves where the aphids had been feeding were cleared using the technique described in 5.2.4. and scanned for areas of phenolic accumulation. The adjacent leaves, which were aphid-free over the course of the experiment, were used as a control. Fifteen insects were recorded on plants of the JL24 (control) and 20 on ICG12991. EPG parameters were compared using t-tests.

Experiment 2

Single aphids were allowed to make a single probe on leaves of ICG12991 until a potential drop (Pd) appeared on the graph indicating an intracellular puncture (n=15).

The same protocol as 5.2.4. was applied to clear leaf tissue for detection of localised areas of phenolics.

Experiment 3

Aphid feeding was recorded on ICG12991 and JL24 for one hour. Sixteen recordings on ICG12991 and 10 recordings on JL24 were compared and aphid feeding sites were analysed for cell damage.

Table 5 1: Summary of main EPG waveforms and their correlation with aphid activity: (e) is extracellular stylet activity and (I) is intracellular stylet activity (after Tjallingii & Hogen Esch, 1993)

EPG pattern	Frequency	Correlation plant tissue	Aphid activity
Np (e)	*	Surface	non penetration
C (e)	Mixed	all tissues	stylet pathway activity[1], including production of both gelling and watery saliva
Pd (I)	*	all living cells	cell membrane puncture
E1 (e)	2-7	unknown	Unknown
E1 (I)	2-7	sieve elements	Salivation (watery saliva)
E2 (I)	0.5-4	sieve elements	phloem ingestion
F (e)	11-19	all tissues	mechanical stylet activity
G (I)	4-9	xylem	xylem ingestion

[1]Stylet pathway activity: Stylet moving in between the cells

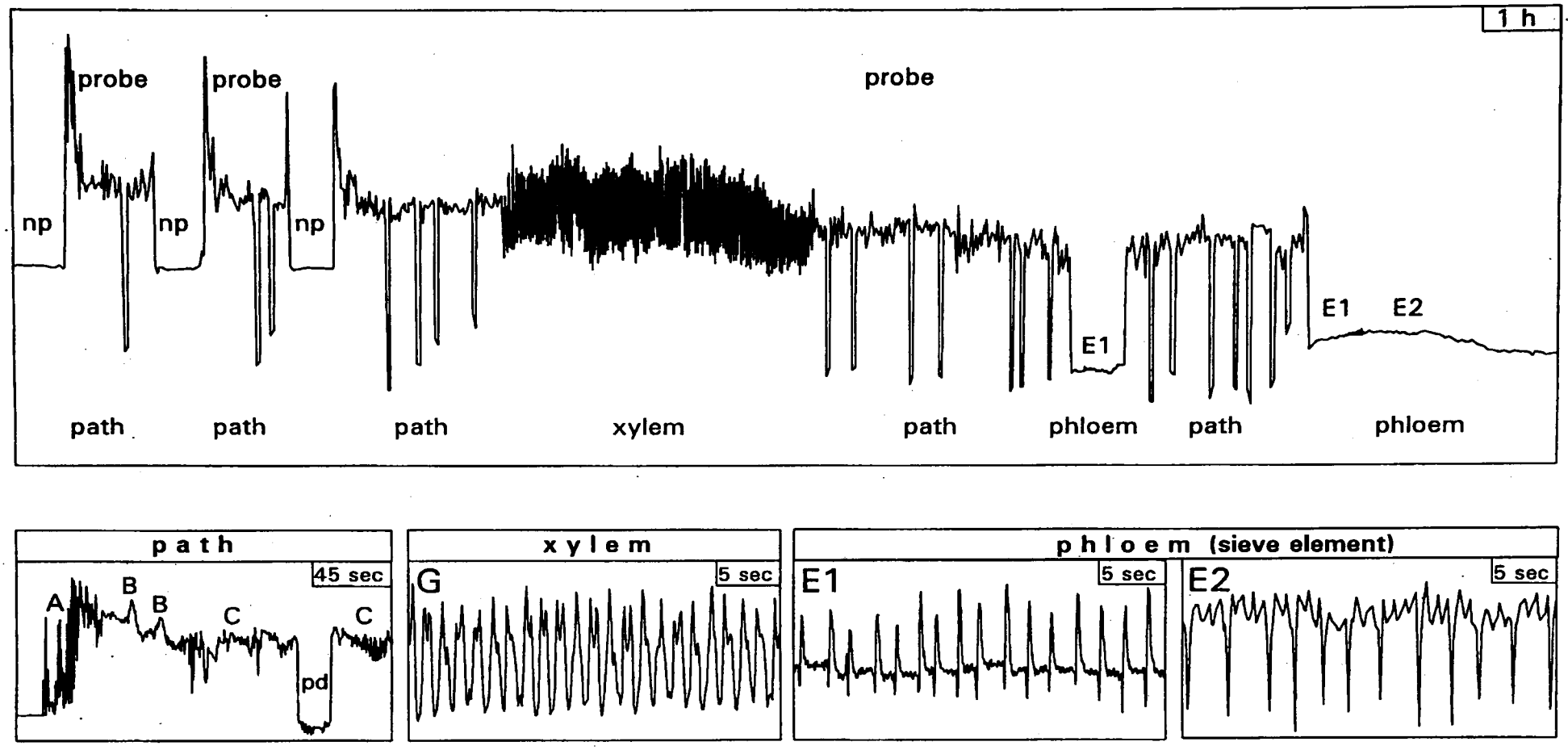


Figure 5 2: Electrical penetration graph of aphid feeding behaviour. Top trace: probing and non-probing alternation. Probe is stylet penetration period, np is non-probing. First two probes contain pathway (path) only, the third probe also includes a xylem phase and two phloem phases: one short with E1 only, the second with E1 followed by E2. Bottom trace: details of each phase with waveform indications; pd = potential drop (intracellular puncture) (Tjallingii, 1990b).

5.3. Results

5.3.1. Aphid behaviour on aphid-susceptible and aphid-resistant groundnut varieties under controlled and glasshouse conditions

No significant differences in aphid probing behaviour were observed between those feeding on ICG12991 and those on JL24 (Table 5.2). In the laboratory at NRI and in the glasshouse in Malawi 7 out of 8 alatae aphid morphs were recorded after 24h on JL24 compared to 2 out of 8 on ICG12991. Aphids made more and shorter probes on plants grown under controlled conditions than those grown under natural conditions in Malawi and the time before the first probe was 3 times longer under glasshouse conditions compared to the controlled conditions.

Table 5 2: Aphid probing behaviour under glasshouse conditions in Malawi and under controlled conditions at NRI, UoG in UK. Plants at the glasshouse were 28 days after planting (DAP) and at the laboratory 14 DAP. Means are represented with S.E.; N=number of replicates.

Glasshouse at ICRISAT Malawi, plants 28 DAP		
Variety	**JL24 (n=8)**	**ICG12991 (n=8)**
Mean time before first probe ± S.E. (sec.)	47 ± 22.5	47 ± 12.6
Mean number of probes ± S.E.	2 ± 0.5	3 ± 0.7
Mean duration of first probe ± S.E. (sec.)	148 ± 48.6	98 ± 44.5

Laboratory at NRI, plants 14 DAP		
Variety	**JL24 (n=8)**	**ICG12991 (n=8)**
Mean time before first probe ± S.E. (sec.)	14 ± 2.4	30 ± 8.2
Mean number of probes ± S.E.	6 ± 1	6 ± 1
Mean duration of first probe ± S.E. (sec.)	61 ± 42.7	55 ± 22.8

5.3.2. Honeydew excretion by *A. craccivora* on leaf and flower tissue of aphid-susceptible and aphid-resistant groundnut varieties in the laboratory

Leaf tissue

Aphids excreted significantly more honeydew deposits on leaf tissue of JL24 than on ICG12991 ($P<0.001$; Student t-test). Moreover on ICG12991, honeydew excretion was almost completely absent over the course of the 48hour period while aphids excreted the first honeydew within the first two hours after plant access on JL24 (Figure 5.3). By the time the first honeydew was collected on ICG12991, 4 hours after aphid access on the plants, approximately 3 deposits were collected on JL24.

The diameter of the excreted honeydew deposits (HD) was also significantly smaller when aphids had fed on ICG12991 than when fed on JL24 ($P<0.001$). The mean diameter of the HDs on JL24 was 1.1 ± 0.01 mm compared to 0.9 ± 0.01 mm on ICG12991 (Table 5.3). First instar nymphs also excreted significantly smaller deposits on ICG12991 than on JL24 ($P<0.001$).

Flower tissue

On flowers of both ICG12991 and JL24, aphids excreted honeydew at an equal rate and of similar size. The first deposits were collected within two hours of plant access by aphids and excretion continued for the rest of the experimental period (Figure 5.4). Due to the ageing of the flowers after 24 hours, a larger variation in recorded number of honeydew deposits was observed.

In addition, the size of the collected honeydew deposits from feeding aphids on the flower tissues did not differ between the varieties (Table 5.3).

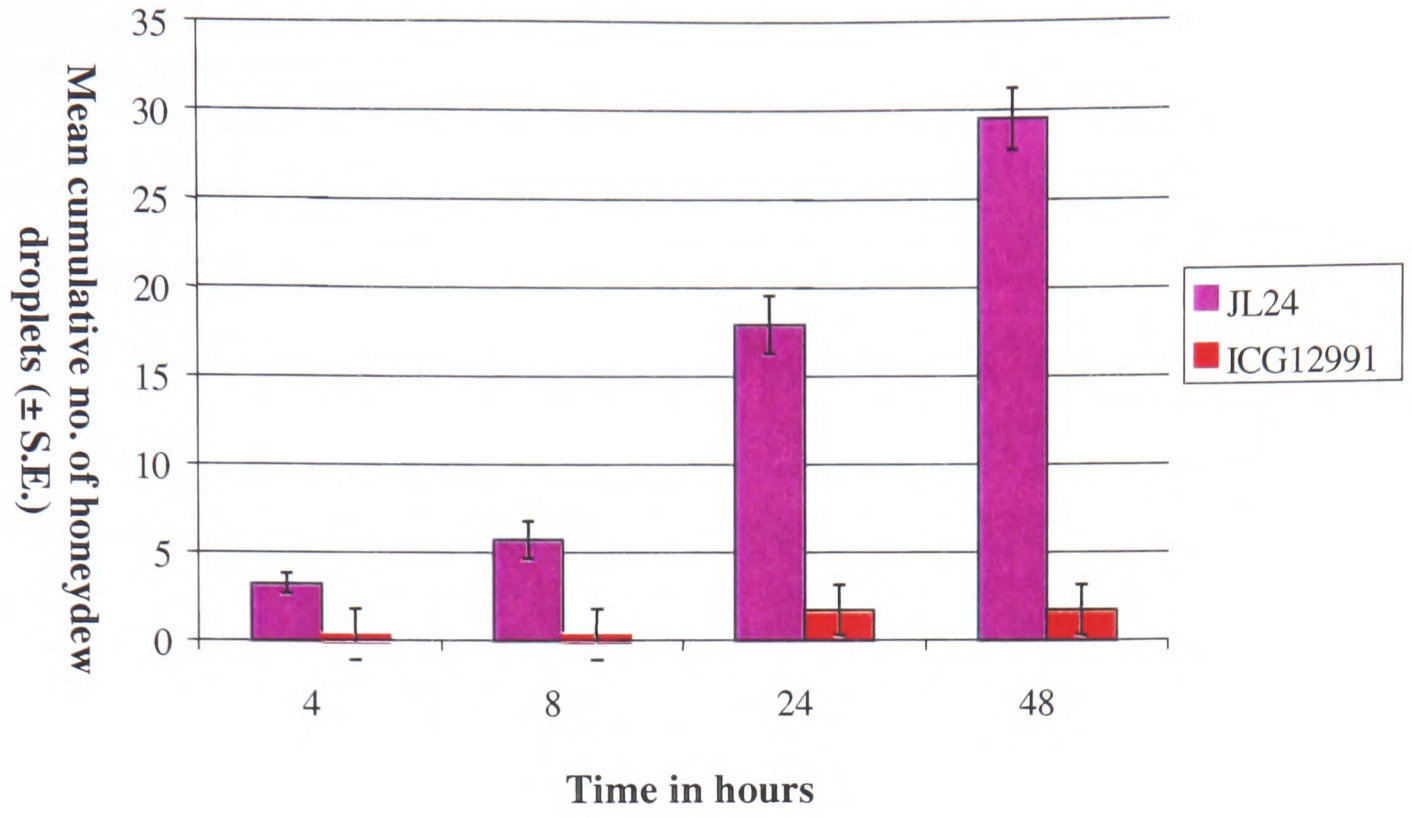


Figure 5 3: Cumulative number of excreted honeydew deposits by adult apterous *A. craccivora* on leaf tissue of groundnut varieties JL24 and ICG12991 over a 24h feeding period.

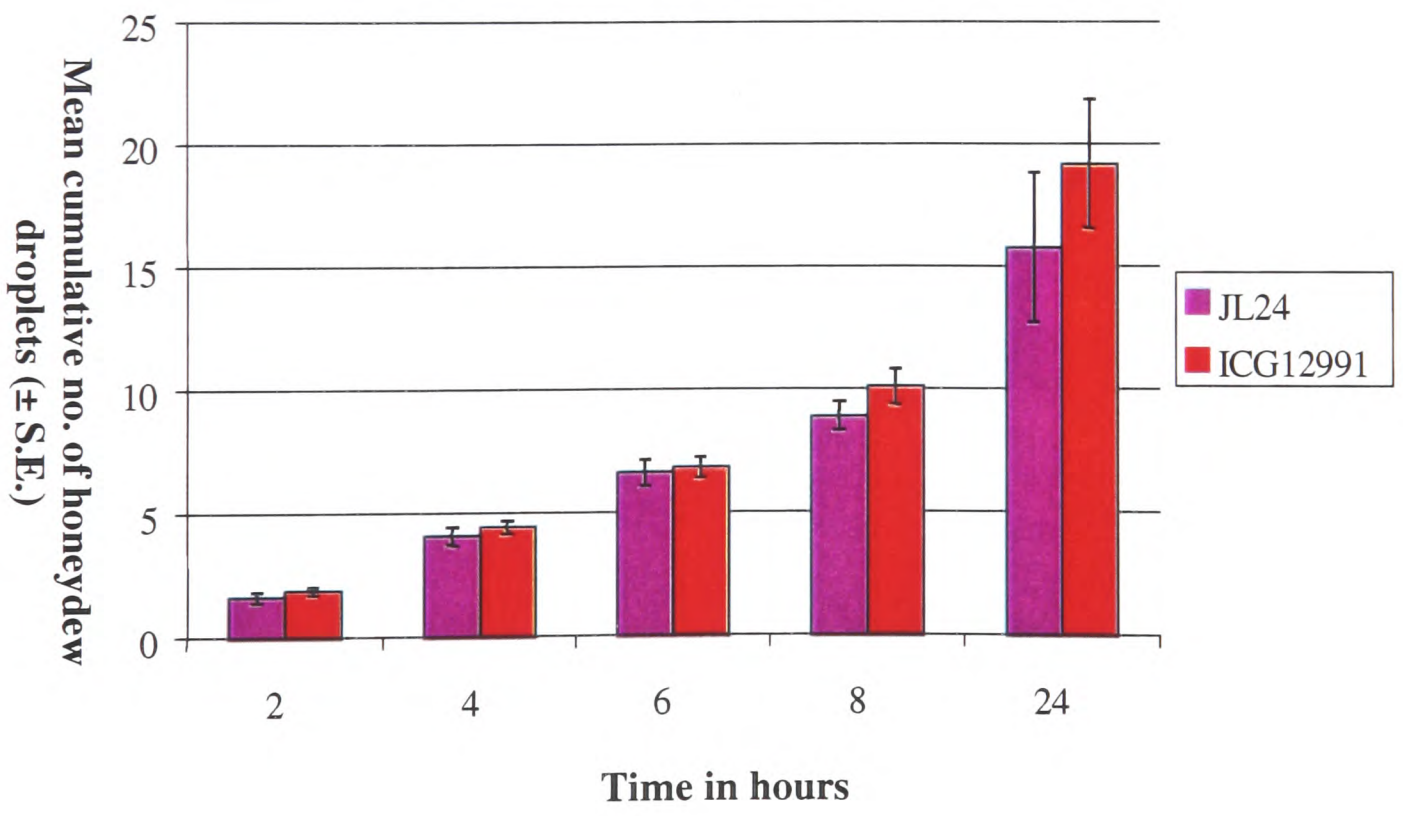


Figure 5 4: Cumulative number of excreted honeydew deposits by adult apterous *A. craccivora* on flower tissue of groundnut varieties JL24 and ICG12991 over a 24h feeding period.

Table 5 3: Diameter of excreted honeydew deposits collected on indicator paper by adults and first instar nymphs of *A. craccivora* on leaf and flower tissue of groundnut varieties JL24 and ICG12991.

Diameter of honeydew deposits by aphids (mm ± S.E.)				
A. craccivora	**Plant tissues of groundnut varieties**			
	Leaves		**Flowers**	
	JL24	ICG12991	JL24	ICG12991
Adult apterous	1.1 ± 0.01	0.9 ± 0.01	1.1 ± 0.01	1.1 ± 0.01
First instar nymphs	0.5 ± 0.01	0.4 ± 0.01	0.5 ± 0.01	0.5 ± 0.01

5.3.3. Localised areas of cell damage on ICG12991 and aphid feeding

Localised collapsed areas on ICG12991 were characterised with the SEM and higher magnification showed that the cells in the area were still under turgor pressure (Figure 5.5a,b). In contrast, mechanical cell damage resulted in collapsed cells (Figure 5.5c). On the control variety JL24, no such areas were identified.

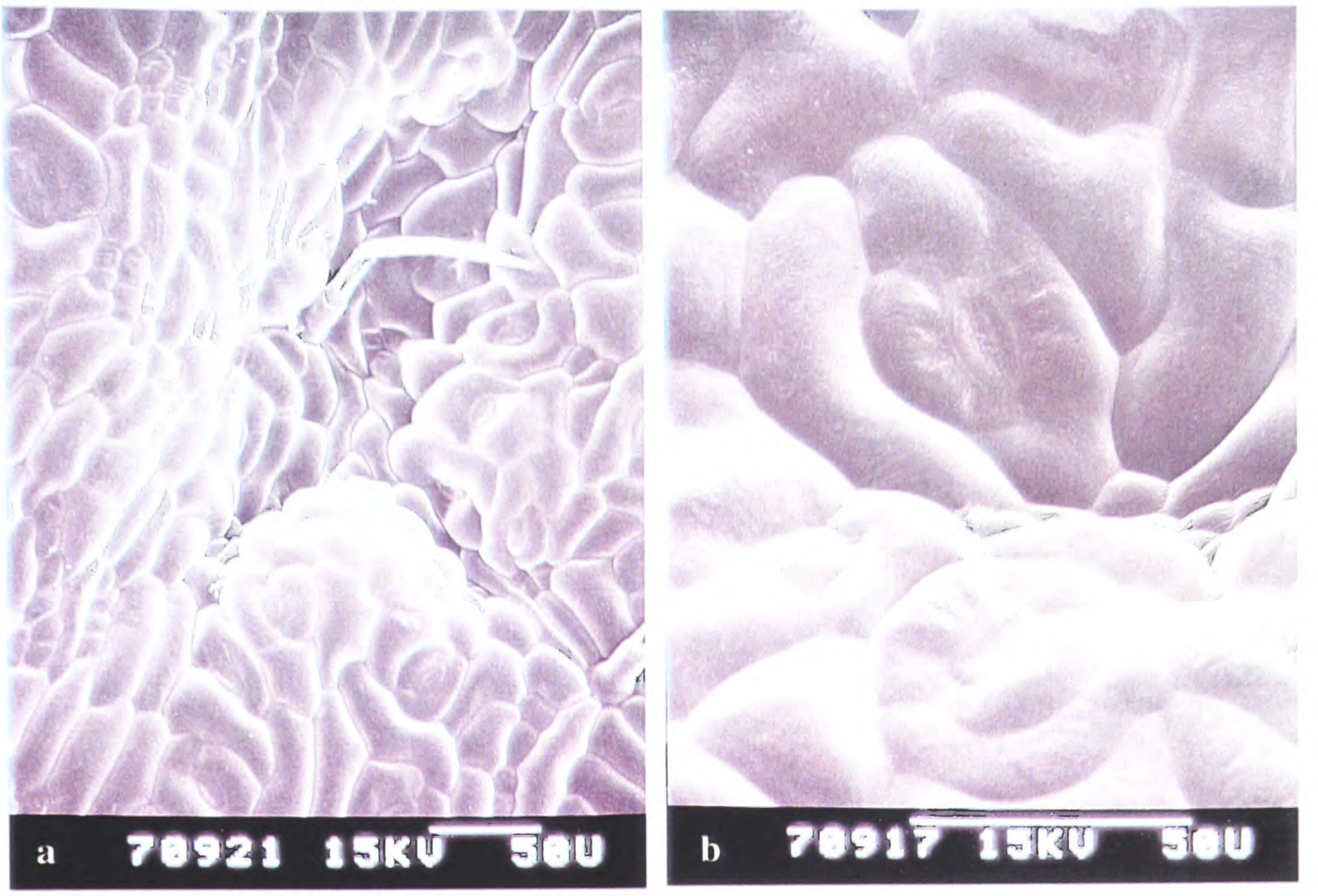


Figure 5 5: Scanning electron micrographs of the abaxial leaf surface of ICG12991 after aphid feeding; a) collapsed area, b) cells in the centre of a collapsed area c) mechanical cell damage. Bar indicates 50µm

5.3.4. Leaf clearing technique and quantification of areas of cell damage

The area of hypothesised accumulated phenolics varied significantly between the varieties and between the modifications of the photographs taken. The modification of the photographs based on a representative colour of the area of cell damage from a colour photograph was less affected by background noise during the process because of the greater contrast using real colours. The areas on both ICG12991 and ICG9723 were twice the size than when the colour picture was first inverted to greyscale (Table 5.4). On ICG12991 the area was around 4 times smaller than on ICG9723. An area of the accumulated phenolics in cleared leaf tissue of ICG12991 is presented in Figure 5.6.

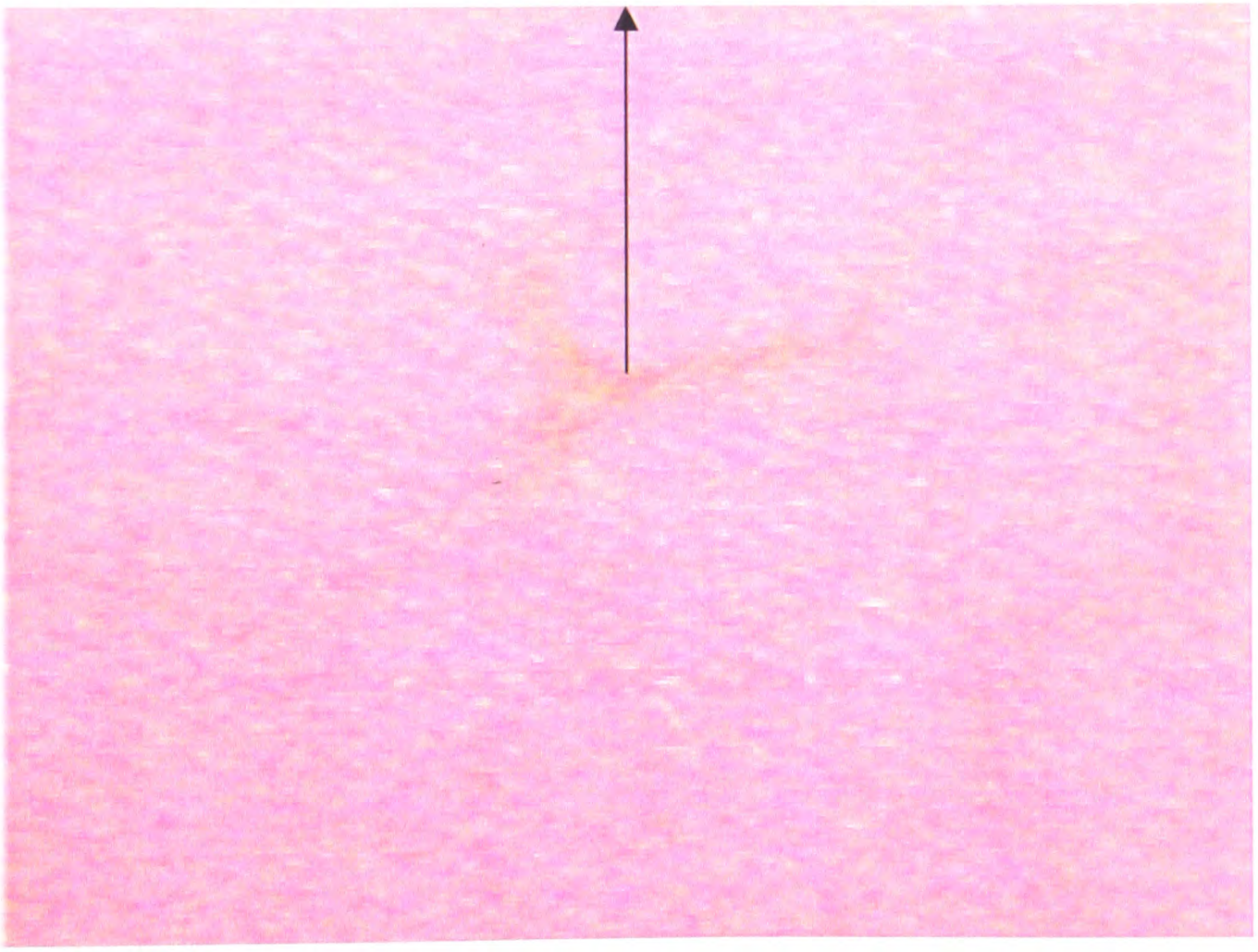


Figure 5 6: Cleared leaf tissue of ICG12991 after aphids had fed on the leaf. (Magnification: 10X12.5).

Table 5 4: Quantified areas of accumulated phenolics (mm^2 ± S.E.) in cleared leaf tissue of groundnut varieties ICG12991 and ICG9723 based on two modifications of colour photographs. First modification was based on inversion to greyscales; second modification was based on a representative colour as seen in colour photographs, Red 138, Green 103; Blue 37.

Quantified area of cell damage associated with aphid feeding on groundnut leaf tissue (mm^2 ± S.E.)

Colour photograph	Groundnut variety	
	ICG12991	ICG9723
Modification 1	0.010 ± 0.0008	0.047 ± 0.0055
Modification 2	0.018 ± 0.0010	0.086 ± 0.0099

5.3.5. Aphid feeding behaviour on aphid-susceptible and aphid-resistant groundnut varieties and cell damage

Experiment 1

Aphids needed almost twice as much time to make a first registered phloem contact (waveform E1 indication salivation into the sieve elements) on ICG12991 compared to JL24 ($P<0.01$), (Table 5.5). The duration of this first salivation period however was similar for aphids feeding on both varieties and lasted approximately 2 min. Within the first hour of a 4h recording period, 43% of the aphids had salivated into the phloem JL24 compared to 8% on ICG12991. Apart from the first E1 registration, other E1 registrations time-recorded from the start of a probe were observed approximately after 17 min and were similar to these on JL24. The transgression of the E1 waveform to an E2 waveform was never observed for aphids feeding on ICG12991, which means that sustained phloem feeding did not occur on this variety within 4 hours. Failure to feed resulted in significantly more pathway activity registration on ICG12991 compared to JL24. On JL24, 87% of the aphids showed periods of sustained feeding. F-patterns were observed for both varieties and 5 aphids showed xylem feeding activity on ICG12991 (data not shown).

In 16 out of 20 samples, localised areas of phenolic accumulation were identified on ICG12991 and 9 showed more than one area that corresponded with the aphid feeding positions. In two control samples of ICG12991, similar areas were identified and on JL24 no such areas were observed.

Experiment 2-3

Single epidermal probes did not result in either phenolic accumulation or localised cell damage.

Areas of phenolic accumulation on ICG12991 were reported in 13% of the samples after one hour of aphid feeding. EPG recordings showed mainly stylet pathway activity and non-probing behaviour as main activities on both varieties. Although 13% of aphids salivated into the phloem of ICG12991, it was not a requisite for cell damage expression. On JL24, 30% of aphids salivated into the phloem.

Table 5 5: EPG parameters for 4hour recordings of the feeding behaviour of *A. craccivora* on two groundnut varieties (JL24 and ICG12991). The mean time aphids spent showing each parameter is expressed in minutes (± S.E.). Significant differences based on Student t-tests are indicated by different letter a,b ($P \leq 0.01$). n= number of replicates.

EPG parameters	Groundnut variety	
	JL24 (n=15)	ICG12991 (n=20)
Np	51.0 ± 14.2^a	85.6 ± 8.2^b
C	83.6 ± 14.8^a	134.0 ± 8.4^b
Duration of 1st E1	1.8 ± 0.3	2.5 ± 0.5
Duration of E2	104.6 ± 18.4^a	0^b
Time to 1st E1 from start of experiment	85.4 ± 13.4^a	157.9 ± 19.2^b
Time to E1 from start of a probe	16.9 ± 2.4	16.5 ± 2.4
% aphids showing E1	93^a	60^b
% aphids showing E2	87^a	0^b

5.4 Discussion

The time taken for *A. craccivora* to make a first registered phloem contact on ICG12991 was greater than on JL24 and the insects were not able to ingest from the phloem sap on ICG12991 within a 4h recording period. Lack of honeydew excretion as an indication of sustained phloem feeding showed that even after 48h, aphids had not fed on leaf tissue of ICG12991 compared to aphids on the susceptible variety JL24. Results from Chapter 2 already showed a shorter aphid development time, a higher fecundity and intrinsic rate of increase (Rm) on JL24 and this is well reflected by the ability to quickly initiate sap uptake and sustain ingestion. Failure to do so on ICG12991 resulted therefore in high mortality rates among aphids, longer development times and reduced fecundity as well as a rapid rejection of ICG12991 within a few hours after plant access (Chapter 2). Additionally, close observations of aphid probing behaviour at the level of the epidermal layer under glasshouse and laboratory conditions did not support the hypothesis that surface chemicals were involved in resistance, such as the resistance reported in raspberry varieties to *Amphorophora idaei* (Robertson *et al.*, 1991). Aphids readily inserted their stylets into the leaf tissue on both the resistant ICG12991 and susceptible JL24. This parameter of aphid behaviour however was not measured for aphids on the EPG system because they were tethered with a gold wire and because of the sensitivity of aphid probing behaviour to pre-treatment (insect handling, food deprivation) (Montllor & Tjallingii, 1989).

The accumulated EPG results and honeydew collection data of feeding aphids on the resistant ICG12991 supported thus far the hypothesis that a resistance factor is encountered after stylet insertion in the leaf tissue and prior to phloem accession. Only 60% of the aphids accessed the phloem on ICG12991 within a 4h recording period, which was reflected by an E1 waveform, compared to more than 90% on the susceptible JL24. On ICG12991 aphids spent more time in the non-penetration phase and pathway activity phase (C waveform) which may indicate restless behaviour from numerous unsuccessful attempts to find a sieve element. Apoplastic factors either constitutive or induced during the stylet pathway towards the phloem sieve elements were more likely to be responsible for the reduced host plant acceptance (Sauge *et al.*,1998).

Those aphids that did access the phloem always took significantly longer to do so from the start of the experiment. However within other probes an E1 was recorded on ICG12991 in a time equally as fast as on JL24. This result may support the absence of a physical barrier. E1 was reported to reflect salivation into the sieve elements, probably helping aphids to prepare the phloem sap before ingestion (E2 waveform) (Helden van & Tjallingii, 1993; Prado & Tjallingii, 1994). Caillaud *et al.*, (1995) hypothesized that E1 reflects an aphid attempt to break plugs like callose or P-proteins in the stylet pathways, which was supported by research on alfalfa resistance to the pea aphid (Girousse & Bournoville, 1994) and resistance in brassica to the cabbage aphid (Cole, 1994). However, when salivating into the phloem, *A. craccivora* did not switch into ingestion. This has important consequences for the acquisition and transmission of the *Groundnut rosette virus* agents. Virus agents can only be acquired from the phloem during ingestion (E2) while transmission can occur during salivation (E1) (Naidu *et al.*, 1999a). This could explain the low infection of *Groundnut rosette assistor virus* (*Luteoviridae*) on ICG12991 and subsequent transmission from ICG12991 to susceptible plants (Chapter 4) and will be further outlined in Chapter 8.

Reduced phloem sap ingestion was often the main feature associated with resistance in EPG studies performed on other aphid/plant interactions and was associated with a feeding deterrent (Campbell *et al.*, 1982; Helden van & Tjallingii, 1993; Cole, 1994; Caillaud *et al.*, 1995; Paul *et al.*, 1996) but phloem ingestion was only observed by one aphid on ICG12991 throughout all experiments. A feeding deterrent or absence of a phagostimulant in the phloem was therefore unlikely to cause resistance in ICG12991.

The data indicated that aphid probing induced a localised plant defence mechanism on leaf and stem tissue of ICG12991. Localised areas of cell collapse and a deposition of phenolics at the aphid feeding sites were identified. The rapidity of cell collapse and/or cell death, the early local accumulation of phenolic compounds (<24h) and the strong association of this induced response with aphid resistance suggested that it can be interpreted as a true hypersensitive response (Alston & Briggs, 1970; Lyth, 1985; Massonié *et al.*, 1981; Miles, 1999). Hypersensitivity has been considered as an important type of an induced defence whereby the plant elicits a response to fungi,

bacteria, virus, nematode, mite or insect attack (Maclean *et al.,* 1974; Agrios, 1988; Fernandes, 1990; Grover, 1995; Gopalan *et al.*, 1996; Low & Merida, 1996; Fritig *et al.*, 1998). The mechanisms involved in generating the HR and ultimately causing resistance have been subjected to intensive research and the most complete picture we have is the HR in response to plant pathogenic bacteria (reviewed in Jabs & Slusarensko, 2000). Many similarities arise when comparing hypersensitivity induced by bacteria, fungi, nematodes and viruses including membrane damage and electrolyte leakage, oxidative burst (production of Reactive Oxygen Species (ROS)) and finally cell collapse and death.

It was hypothesized that the HR induction on ICG12991 was linked with the aphid stylet pathway activity in search for the phloem because salivation and ingestion into the phloem were not always recorded when the response was expressed. Detailed observations with the scanning electron microscope showed that the epidermal cells were still under turgor pressure and therefore considered as being still functional. This was supported with the observation that a single (epidermal) cell puncture and a short feeding access period did not generally result in cell collapse or detection of phenolics after the tissue clearing procedure. It rather suggested that cell(s) underlying the epidermis underwent a structural change or collapsed, which then caused the epidermal cells to sink in, showing "dips" on the abaxial surface of the leaves where stylet insertion occurred and white lesions on the adaxial surface. A similar observation of an HR was recorded on barley infected by the fungus *Erysiphe graminis* var. *hordei*, where resistance to infection was dependent on the speed of cell collapse in the mesophyll cells underlying the haustorium-penetrated epidermal cells (White & Baker, 1954).

The induced plant response was not expressed after aphids had been feeding on the flowers of ICG12991. Aphids were also not deterred from feeding on the flowers of ICG12991. The number of deposits and time to excrete the first deposit did not differ on this variety compared to aphids feeding on the highly susceptible JL24. Although the concentration of phloem compounds may vary between tissues, the presence of phloem deterrents will be further examined and outlined in following chapter (Chapter 6).

Many similarities arose when comparing EPG data of *A. craccivora* on ICG12991 and *Myzus persicae* on the resistant 'rubira' variety of peach (Sauge *et al.*, 1998). The number of probing and non-probing times were significantly higher than on control varieties, time to reach the first E1 was longer (but not significant), and 35% of the aphids did not succeed in producing E2 over 8h. More importantly, red or yellow spots were reported to be induced on rubira by feeding aphids (Sauge *et al.*, 1998). However they did not make an attempt to correlate their results with the induced response. The researchers relate the resistance to apoplastic factors either constitutive or induced during stylet pathway activity to be responsible for their observations. Based on the results from this study and in Sauge *et al.*, (1998) it is hypothesized that aphid probing may induce physiological changes which may or may not result in the expression of a hypersensitive response and these changes may be detected in the apoplastic fluids during stylet pathway activity. More research is required to describe hypersensitivity in groundnut and this could become an ideal model to better understand different aspects of HR induced by plant sucking insects.

To test this hypothesis further it needed to be ascertained that feeding deterrents or constitutive deterrents associated with leaf tissue of ICG12991 would play a role in reduced host plant resistance. The ability of aphids to feed from flowers suggested that such chemicals could be absent in these tissues. It was therefore decided to investigate chemicals in these tissues and their effect on aphid feeding behaviour.

CHAPTER 6

THE DEVELOPMENT AND APPLICATION OF ARTIFICIAL FEEDING SYSTEMS TO INVESTIGATE THE INFLUENCE OF GROUNDNUT EXTRACTS ON THE FEEDING BEHAVIOUR OF *A. CRACCIVORA*

6.1. Introduction

To date it is unknown how aphids and other phloem-feeding insects locate their feeding sites, the phloem sieve elements (PSE). Intercellular and intramural[2] stylet pathway activity while random sampling of cells has been proposed and can offer a plausible explanation (Helden van & Tjallingii, 1993). Puncturing cells with their stylets, aphids may ingest sap to sample cell contents by the gustatory organs of the epipharyngeal organ. However, on nonhost plants and resistant plants, aphids may still locate the PSE before they abort their probing behaviour and leave the plant and often a chemical is linked with phloem sap contents, which deters the aphid from ingestion (Nault & Styer, 1972; Schoonhoven & Derksen-Koppers, 1976; Argondona *et al.*, 1980; Herrbach, 1985; Mittler, 1988; Harrewijn, 1990; Niemeyer, 1990; Helden van, 1995). The question therefore still remains unanswered what role the chemical cues from the peripheral tissues play in the host-plant selection process of aphids. On the aphid-resistant groundnut variety ICG12991, *Aphis craccivora* was significantly delayed in making a first registered phloem contact as compared to feeding on a susceptible one, JL24, which was measured by an electrical monitoring graphs system (EPG, Chapter 5). The observation implied that a resistance factor associated with the peripheral tissues in ICG12991 was involved either directly or indirectly. The delay may result from a mechanical barrier, such as thickened epidermal or sclerenchyma leaf tissues, or a chemical barrier. However, *A. craccivora* was able to reach the PSE on ICG12991 in a

[2] Intramural path: pathway inside the cellular space bordered by the cell walls, but clearly outside the plasmallemma and therefore extracellular with respect to the living cell.

time similar to that on JL24 when measured from the time to make any but the first probe (Chapter 5); therefore such barriers were not investigated.

To investigate the role of plant chemicals on aphid feeding behaviour, artificial diets are often used. Phloem-feeding insects and their modified mouth-parts as a sucking feeding apparatus make it a difficult group of insects to design a representative feeding bioassay as opposed to leaf-chewing insects such as caterpillars. Mittler & Dadd developed the most commonly used feeding bioassay in 1962, which was based on a feeding sachet composed of a parafilm membrane overlaying a liquid diet. The system was designed to provide an environment which would enable food uptake, oxygen consumption, salivation and egestion by phloem-feeding insects (Hertel & Kunkel, 1976; Harris & Bath, 1973; Harris, 1977). However, the mesophyll area between epidermis and the phloem sieve elements is likely to play a significant role in early steps of host plant selection by phloem feeders. Therefore a bioassay was developed based on a solid probing medium such as agar, starch or agarose (Urbanska *et al.*, 1998), which originated from the early work of Davidson (1923) and Staniland (1924). Their design as feeding or probing bioassay had mainly been applied to detect aphid salivary enzymes and although many enzymes were detected this way (Davidson, 1923; Staniland, 1924; Ma *et al.*, 1990; Miles & Harrewijn, 1991; Peng & Miles, 1991; Urbanska *et al.*, 1998), it could be applied to look more in detail to aphid feeding behaviour during pathway activity. By mixing plant-based or synthetic chemicals into the probing media, and examining the number of probes, size and other characteristics, changes in feeding behaviour may be detected. The bioassay is specially advantageous when a resistance factor other than a phloem based chemical or volatile was likely to cause plant resistance, which was proposed for the aphid resistance in groundnut variety ICG12991. Therefore, the bioassay based on agarose was applied to investigate whether aphids could detect chemicals mixed in agarose gels that subsequently alter aspects of their feeding behaviour. First, known aphid feeding stimulants and deterrents were added to diets either as a solution in parafilm sachets, modified from Mittler & Dadd (1962), or in agarose substrates and the effects of these chemicals on aphid feeding was recorded. Once baseline data were collected, extracts of plant tissues of a

susceptible (JL24) and resistant variety of groundnut (ICG12991) were incorporated into the diets.

6.2. Materials and Methods

Attempts to locate aphid stylets in plant tissue to study aphid feeding behaviour in natural systems were unsuccessful due to technical difficulties. This has led to the design of artificial feeding systems based on literature research.

6.2.1. Aphids

6.2.1.1. Parameters of aphid feeding on artificial diets

When most aphid species probe substrates, they leave behind gelling saliva, known as the stylet sheath (Miles, 1999). Each sheath that is observed in an artificial diet after aphids had fed on it is referred to as a probe and the number of probes, its branching pattern and size were used as parameters of aphid feeding behaviour in the bioassays. The length of each detected probe was measured or estimated with a graticule (1mm/0.01 div; Graticules Ltd, Tonbridge Kent, UK) and classified as a small probe (≤ 50μm), medium sized probe (50-100μm) or a long probe (>100μm).

6.2.1.2. Aphids selected for the feeding experiments

An aphid population of *A. craccivora* originating from Uganda was maintained on a susceptible groundnut variety Malimba under controlled conditions at NRI (Chapter 2). Preliminary aphid feeding experiments, of which the results are not included, showed that confining ten aphids (apterous morphs) on a diet for an 18h feeding period was suitable and practical to locate and measure a representative sample of probes. It was required to reduce the number of aphids from 10 to 5 per replicate when plant-extracts were applied in the diets to collect accurate data. (6.2.4.4 and 6.2.4.5). It was decided not to count the number of probes on sub-sections of the diets for practical reasons.

Aphids that were used in the experiments were aged as approximately 24h after their last moult and deprived or not deprived of food as will be specified for each experiment (6.2.4).

6.2.2. Plants

Water extracts of different plant tissues of groundnut varieties were applied in the diets to examine whether chemicals associated with these varieties would have an effect on aphid feeding behaviour. The varieties selected were ICG12991, an aphid-resistant variety and JL24, an aphid-susceptible variety (Chapters 2-5). The plants were grown under artificial conditions at NRI (Chapter 2).

6.2.3. Feeding bioassays: description and design of two systems

6.2.3.1. Bioassay based on a liquid diet in a parafilm sachet

Mittler & Dadd described the technique in 1962, in which the diet is presented in an envelope composed of two opposing membranes of Parafilm M^R. A square of parafilm was stretched into a thinner membrane in two directions at right angles to each other and disposable latex gloves (Glovco ^{TM}) were worn to minimise membrane contamination. The membrane was draped over one side of a cylinder cut from a pyrex tube (inner diameter 2cm, height 2cm) and the edges were abraded to prevent laceration of the membranes. With a sterile pipette, 0.3 ml of artificial diet was dispensed on the membrane. A second parafilm membrane was then stretched over the diet, spreading it out between the two membranes. The aphids were enclosed in the cylinders (containers), (Figure 6.1a). All replicates were placed in a black box with light entering only from the top to attract aphids to the membrane surface and to stimulate feeding on the diets. After the 18h feeding period, the aphids were removed and a 0.1% safranin solution was carefully injected into the diets between the two membranes to stain the sheaths red because of bindings with the sheath-proteins (Figure 6.1b). The upper membrane was then carefully removed with tweezers and the outer surface of the lower membrane was carefully rinsed with distilled water to remove the excess of safranin. In order to count and measure the length of the probes, the cylinder was positioned in a way that the inner surface of the membrane could be observed under a

stereomicroscope, magnification 20X50 (Figure 6.1c). The image as observed under the microscope is presented in Figure 6.3a.

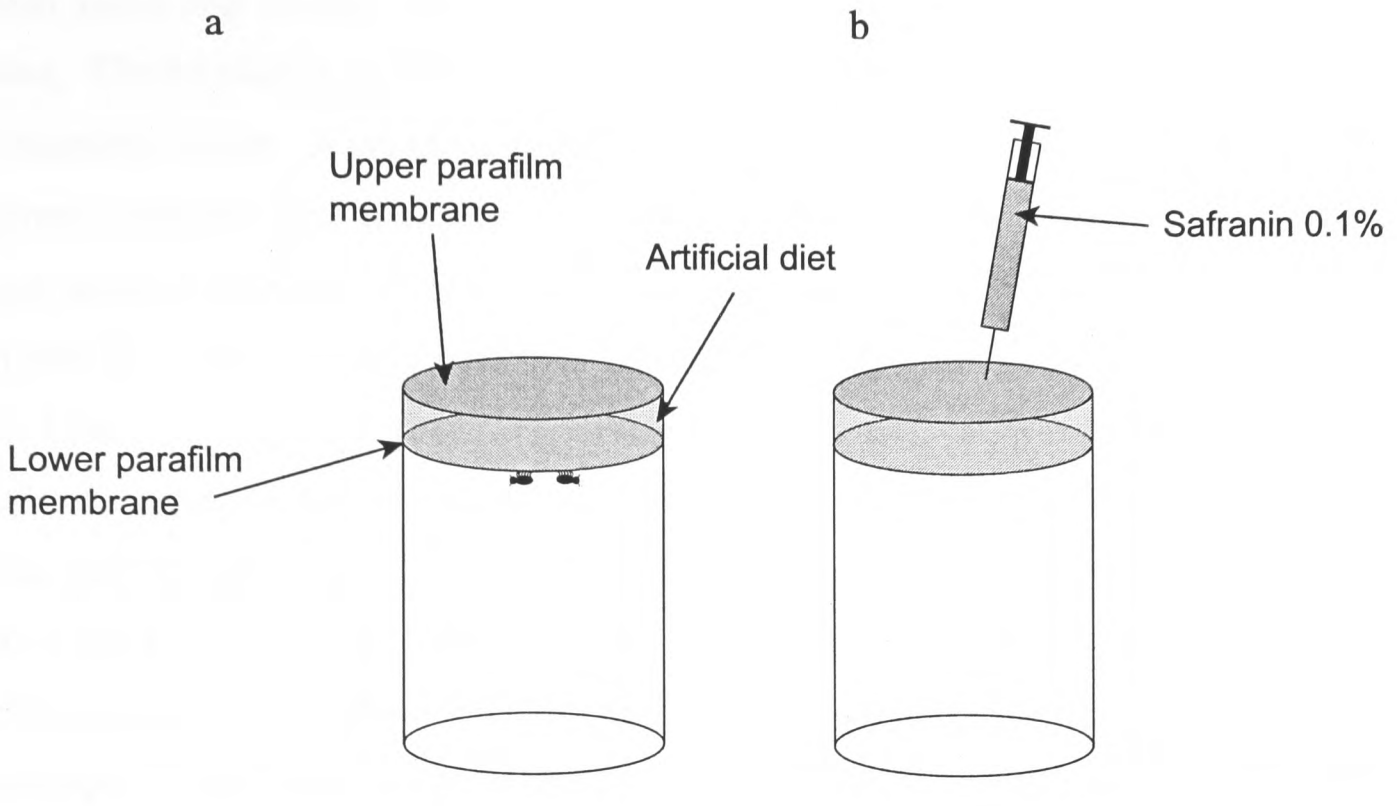


Figure 6 1: a) Aphid feeding bioassay in which the diet is presented in a sachet composed of two parafilm membranes; b) injection of safranin 0.1% in the diet to stain the probes in the lower parafilm membrane by feeding aphids; c) visualisation of probes under 1000X magnification after removal of the upper membrane.

6.2.3.2. Bioassay based on an agarose gel as diet

Agarose gels (1.2% w/v) as probing substrate were prepared by dissolving agarose in distilled water and heating the solution on maximum power in a microwave for 2-3 minutes. The solution was left to cool to approximately 40°C while continuously stirred on a magnetic stirrer. A parafilm membrane was previously stretched over one side of the pyrex containers and positioned on a sterile Petri-dish (Figure 6.2a). The cooled agarose solution was then divided over pyrex containers (2ml/ container) and left for a minimum of 15 min. for gel formation. The gels were used immediately as insect diets in the bioassays (Figure 6.2b). Ten aphids were confined under a second container, sealed with parafilm and positioned on top of the gel-containing cylinder for an 18h feeding period (Figure 6.2c). Replicates that were tested simultaneously were placed inside a black box with light entering from the bottom to stimulate feeding. After the 18h feeding period, the aphids were removed and the gels were observed under a stereo microscope to count the number of probes and to measure the length of each individual probe (Figure 6.2d). The latter measurement was based on estimation with a graticule in the ocular of the microscope rather than accurate measurements because the probes were directed downwards into the diet (Figure 6.3b).

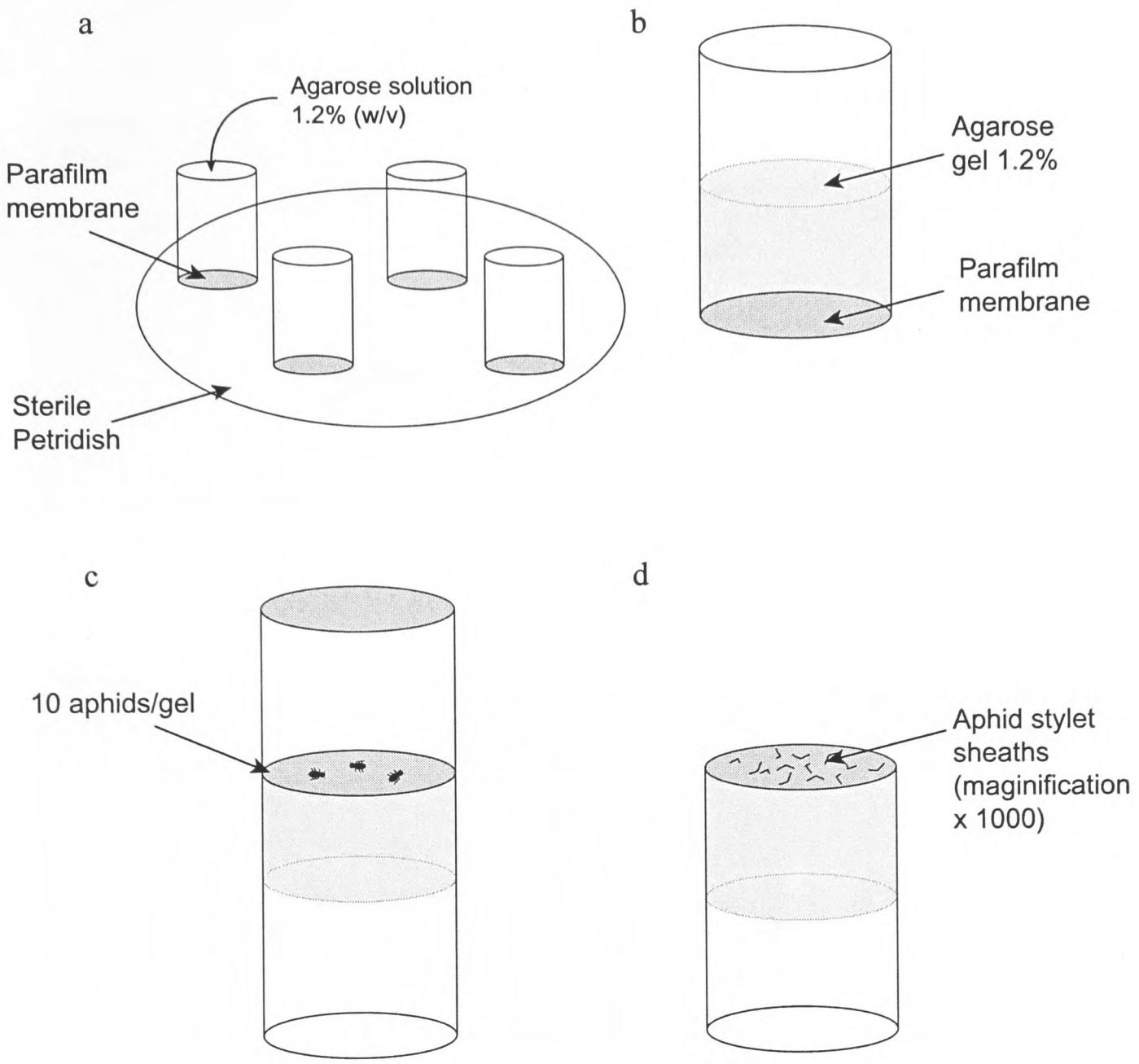
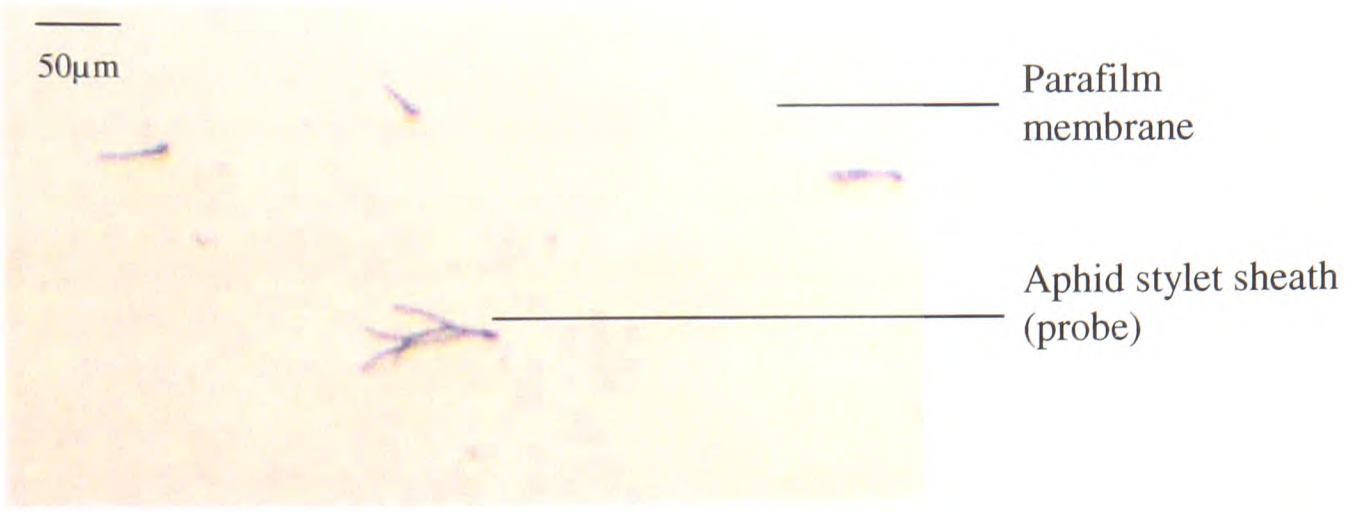


Figure 6 2: a,b) Aphid feeding bioassay in which the diet is presented as an agarose gel in feeding chamber (1.2% w/v); c) Aphids enclosed on the diets; d) direct observation of aphid-probes under 1000X magnification binocular microscope.

a)



b)

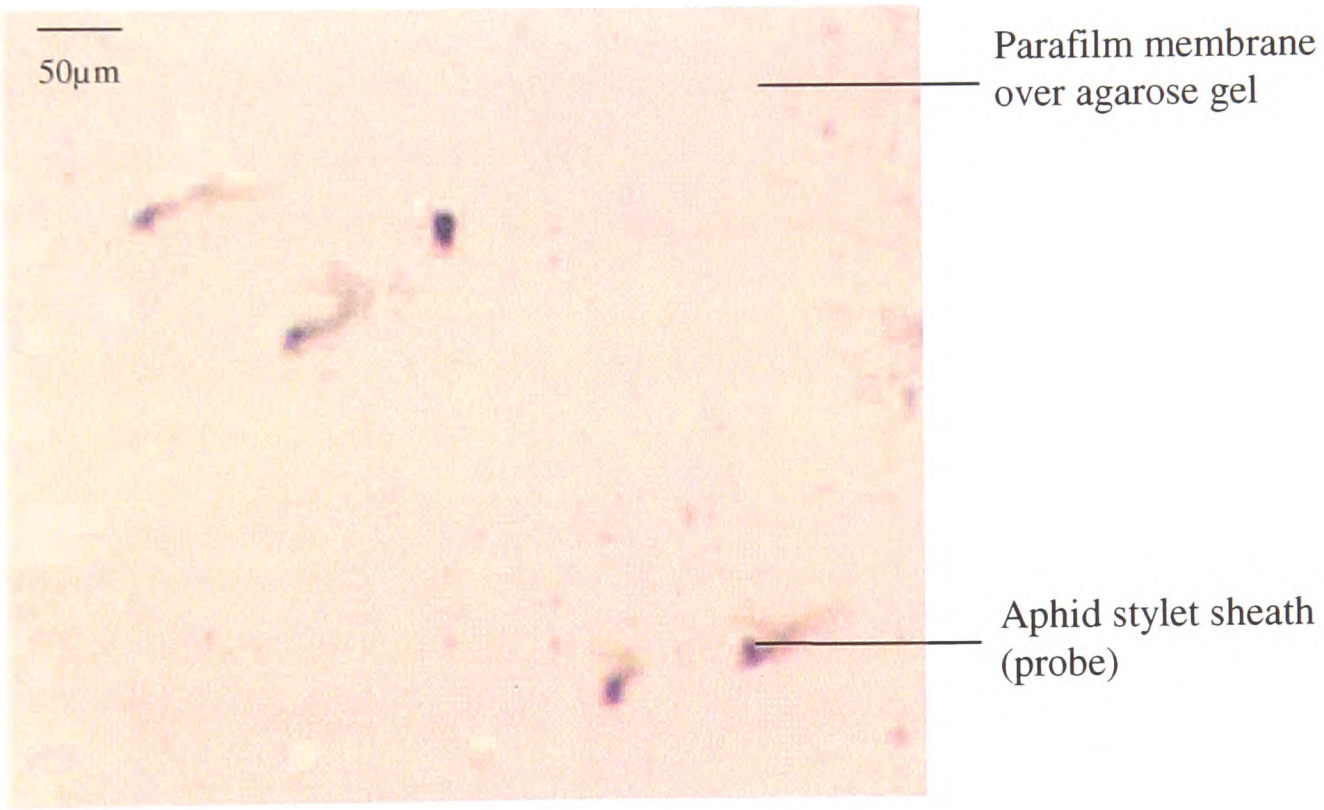


Figure 6 3: Aphid probes on artificial diets as observed under microscope (20x50); a) diet as a liquid solution in parafilm sachet; aphid probes were stained in safranin 0.1%; b) diet as agarose gel (w/v 1.2%), aphid probes as observed under microscope. Probes directed downwards into the gel.

6.2.4. Validation of the feeding bioassays by adding aphid feeding stimulants and deterrents to the diets

To validate the feeding bioassays to study the feeding behaviour of *A. craccivora*, known aphid feeding stimulants and deterrents were incorporated into the diets. The effect of the chemicals on *A. craccivora* was separately evaluated in the sachets and gels.

To incorporate the chemicals in the gels they were added to cooled agarose solution (1.2% w/v). The mixtures were then stirred for 15-20 sec on a magnetic stirrer and distributed among over the pyrex containers as described earlier (6.2.3.2).

Sucrose was first added because a sucrose solution with a concentration ranging from 10-35% is a known aphid phagostimulant and can stimulate aphid feeding (Mittler & Meikle, 1991). A 20% concentration was chosen to incorporate into the diets.

Following a feeding stimulant, two different aphid antifeedants were added to the diets because of their different mode of action against plant sucking insects.

Pymetrozine (Plenum ®), ((E)-4,5-dihydro-6-methyl-4-(3-pyridylmethyleneamino)-1,2,4-triazin-3(2H)-one) is a selective compound with activity specifically against homopterous insects (Flueckiger *et al.*, 1992a). It causes the insects to stop feeding irreversibly and death by starvation occurs after 1-3 days. Pymetrozine acts by ingestion as well as by contact (Novartis, 1998).

The active compound of neem-oil, which is isolated from the seeds of *Azadirachta indica* is azadirachtin (tetranortriterpenoid) (Butterworth & Morgan, 1968). Azadirachtin is a natural plant defence chemical affecting feeding through both chemoreception (primary antifeedancy) and ingestion (secondary antifeedancy). It affects the insects' ecdysteroid and juvenile hormone titres and the endosymbionts from its guts. Azadirachtin is active against a range of insects, nematodes and fungi. (Reviewed in Mordue & Blackwell, 1993; Heuvel van den *et al.*, 1998). Even the odour

of neem has been described as disrupting normal feeding behaviour of the green leafhopper *Nephotettix virescens*, Distant (Homoptera, Fam. Cicadellidae) on rice plants (Saxena & Kahn, 1986). The neem-oil was provided by the Royal Botanical Gardens, Kew, (Surrey, UK) but the concentration of the active ingredient azadirachtin was not known.

6.2.4.1. Sucrose 20%

Parafilm sachets

A 20% sucrose solution in distilled water was prepared and distilled water only was the control diet. Aphids of the Ugandan population were confined immediately on the diets and the experiment was replicated nine times (n=9). The experiment was repeated for aphids originating from a Malawian population (n=7).

Agarose gels

The agarose solution (1.2% w/v) was mixed with 20% sucrose (w/v). Agarose gels without the added sucrose were control diets. The aphids were not deprived of food and 10 replicates for each treatment were compared on number and length of identified probes.

Because no information about aphid feeding on an agarose substrate was available in the literature, migratory aphid morphs were also selected to feed on the agarose gels. Migratory aphid morphs were selected as winged aphids in the top of the insect cages. The different aphid morphs were tested independently of each other because the aim was to compare the effect of sucrose-agarose diets versus control diets.

6.2.4.2. Pymetrozine

As no information on aphid feeding on pymetrozine in artificial diets was available, the effect of 100, 300, and 1000ppm was first evaluated in both the sachets and gels. Lower concentrations of 25 and 75ppm were tested in a second series of experiments due to the strong observed effects on the higher concentration (100-1000ppm). Aphids were deprived of food for 3h prior the start of the experiment.

Parafilm sachets

Six replicates were analysed for the higher concentrations of pymetrozine in a 20% sucrose (w/v) solution with 20% sucrose only as a control diet. In contrast, only 3 replicates were compared on the effect of lower concentrations of pymetrozine on aphid feeding

Agarose gels

The observed effects of pymetrozine on aphid feeding in sachets were evaluated in gels. Six replicates were first analysed to evaluate the effect of higher concentrations of pymetrozine in agarose gels on aphid feeding behaviour. Three replicates were analysed in a second experiment to evaluate the lower concentrations of pymetrozine. Untreated agarose gels were control diets.

6.2.4.3. Neem-oil

Azadiractin was first extracted by gently heating neem-oil into water with Tween 20% to dissolve the oil. This method was unreliable and therefore azadirachting was extracted in ethanol (96%). As the concentration of azadirachtin in the neem-oil provided was unknown, 30% neem-oil was extracted in ethanol under continuous stirring for approximately one hour. Aphids were food-deprived for 3h prior to the experiments.

Parafilm sachets

The supernatant of the extract was further diluted into a previously prepared sucrose 20% solution to a final 0.05% ethanol concentration. The concentration of neem was 0.022%. The control treatments were 20% sucrose solution with an ethanol concentration of 0.05% and sucrose 20% only. Five replicates for each treatment were analysed (n=5).

Agarose gels

The supernatant of the neem-oil extract in ethanol was added to the agarose solution at 40°C to a final neem concentration of 0.022% (0.05% EtOH). The experiment was replicated 7 times (n=7).

6.2.5. Diet containing plant extracts from groundnut and the effect on aphid feeding behaviour in bioassays

6.2.5.1. Extracts of leaf tissue

Water extracts of the youngest developing leaflets of groundnut varieties, JL24 and ICG12991 were prepared. The plants were in their 4th leaf-stage and approximately 14 days after planting. The youngest leaflets of individual plants were boiled together for 15 min (1g/5ml), left to cool and filtered through Whatman No.1 filter paper followed by Whatman 0.45µm syringe filter (Powell & Hardie, 2001). The extract was stored at -20°C in aliquots of 5ml.

Only 5 apterous aphids, which were deprived of food for 3h, per replicate and per treatment were confined on the diets for 18h.

Parafilm sachets

In a first experiment, the extracts of JL24 and ICG12991 were used as diets in the feeding bioassay for direct comparison (n=10). Controls were included such as distilled water (n=5) and 20% sucrose (n=5).

Also a 10X dilution of the extracts in distilled water was prepared as diet to check the activity of the diluted extract on the feeding behaviour of *A. craccivora*. This second experiment was necessary because adding extracts in agarose gels would automatically dilute the extract (see next section, agarose gels). The results of the undiluted extracts on aphid feeding are presented.

An additional experiment was conducted to compare the extracts of JL24 with ICG12991 at different dilutions. A fresh extract of groundnut leaves was prepared in water and rotavapored to dryness. The dryweight was measured and re-dissolved in distilled water to a concentration of 1mg/1ml. In separate experiments, the effect of the

extract of both varieties and its dilutions (10X, 100X and 1000X) on aphid feeding behaviour were directly compared.

Agarose gels

To prepare agarose gels containing water extracts of leaf tissue of JL24 and ICG12991, 1ml extract was added to 9ml agarose solution (1.2% w/v). Therefore the extract was diluted 10 times but its effect on aphid feeding was first evaluated in feeding sachets (see previous section). The experiment was replicated 8 times (n=8).

6.2.5.2. Extracts of flower tissue

Flower stems of groundnut varieties JL24 and ICG12991 were collected and stored separately at –80°C. For every 5 ml of distilled water, 0.3g of flowers were boiled for 15 minutes, minimising evaporation. The extract was allowed to cool and then filtered through Whatman No.1 filter paper and again through a 0.2µm syringe filter (Puradisc^{TM} 25 AS). The extracts were stored at –20°C in aliquots of 5ml.

Parafilm sachets

In a first series of experiments, the extracts as prepared (0.3g/5ml) were used as aphid feeding diet (n=9). Controls included distilled water only (n=8) and a 20% sucrose solution (n=9). As described for leaf extracts, a 10X dilution for each diet was prepared (n=7). Controls included distilled water (n=3) and sucrose 20% (n=3). This experiment was again necessary to ensure activity prior to applying the extracts in agarose diets.

Agarose gels

Flower-extract-containing gels were prepared as described for leaf extracts (6.2.5.1) and the experiment was replicated 9 times (n=9).

6.2.6. Statistical Analysis

Data on number of probes and number of offspring recorded after 18h were analysed with non-parametric analysis, Mann-Whitney *U* test and Kruskal-Wallis (Zhar, 1984). The proportion of small (≤ 50µm), medium sized (50-100µm) and long probes

(>100μm) on the various treatments were compared with LOGIT ANALYSIS in GENSTAT software package (6th Edition).

6.3. Results

6.3.1. The effect of stimulants and deterrents in artificial diets on the feeding behaviour of *A. craccivora*

6.3.1.1. Sucrose 20%

Sucrose showed a significant effect on the behaviour of *A. craccivora*, either as a feeding stimulant in the sachets or parturition stimulant in the gels.

Parafilm sachets

Aphids probed significantly more on sucrose solutions than on distilled water ($0.01<P<0.05$, Mann-Whitney *U* test) and this was consistent for both aphid populations (Table 6.1a,b). Although length of measured probes from Ugandan aphids did not differ between both diets, Malawian aphids had probed significantly longer on the sucrose solution (50-100μm; $P<0.05$). Sucrose had no influence on the number of offspring over an 18h feeding period.

Agarose gels

In contrast to a feeding stimulatory effect of sucrose in parafilm sachets, this effect was not always reflected in agarose-sucrose gels except for the Ugandan migratory aphid population (Table 6.2a-d). However, significantly more offspring were observed on the sucrose-agarose gels at the end of the experiment than on the control gels ($P<0.01$; Mann Whitney *U* test).

These observations were consistent for different aphid morphs feeding on agarose-sucrose gels.

Table 6 1: Number and length of probes from 10 aphids confined on diets in parafilm sachets for 18h; Ugandan aphids (Table 6.1a), Malawian aphids (Table 6.1b). Sachets contained sucrose 20% solution or distilled water only (control).

a) Ugandan aphids 10 aphids/sachet	**Distilled water**	**Sucrose 20%**
n	9	9
No. of probes/sachet	23[1]	40[2]
No. of nymphs /sachet	7	10
[1,2] Significant differences across columns: $P<0.05$ (Mann-Whitney U test)		
% Probes ≤50 μm	61 ± 3	57 ± 4
% Probes 50-100 μm	25 ± 3	29 ± 4
% Probes >100 μm	14 ± 3	15 ± 4

b) Malawian aphids 10 aphids/sachet	**Distilled water**	**Sucrose 20%**
n	7	7
No. of probes/sachet	20[1]	63[2]
No. of nymphs/sachet	2	4
[1,2] Significant differences across columns: $P<0.01$ (Mann-Whitney U test)		
% Probes ≤50 μm	64 ± 3[a]	47 ± 4[b]
% Probes 50-100 μm	26 ± 4[a]	39 ± 4[b]
% Probes >100 μm	10 ± 3[a]	14 ± 4[a]
[a,b] Significant differences across columns $P<0.05$ (Logit Analysis)		

Table 6 2: Number and length of probes from 10 aphids confined on diets as agarose gels (1.2% w/v). Aphids selected for the experiments were Ugandan apterous (Table 6.2a), Ug. Apterous migrants (Table 6.2b), Malawian apterous (Table 6.2c) and Malawian apterous migrants (Table 6.2d). Migrants were identified as walking around in the cages). Gels were either mixed with sucrose (20%) or not (control); n indicates number of replicates

Ugandan aphids

a) Apterous 10 aphids/gel	Agarose	Agarose – sucrose
n	10	10
No. of probes/gel	97	84
No. nymphs/gel	3[1]	12[2]
[1,2] Significant differences across columns, $P<0.01$ (Mann-Whitney U test)		
% Probes ≤50 µm	15 ± 3	8 ± 4
% Probes 50-100 µm	58 ± 4	57 ± 4
% Probes >100 µm	27 ± 4	35 ± 4

b) Apterous migrant 10 aphids/gel	Agarose	Agarose – sucrose
n	8	8
No. of probes/gel	95[1]	143[2]
No. nymphs/gel	4[1]	21[2]
[1,2] Significant differences across columns, $P<0.05$ (Mann-Whitney U test)		
% Probes ≤50 µm	8 ± 4	7 ± 3
% Probes 50-100 µm	56± 4	52 ± 4
% Probes >100 µm	36± 4	41± 4

Malawian aphids

c) Apterous 10 aphids/gel	Agarose	Agarose – sucrose
n	10	10
No. of probes/gel	130	104
No. nymphs/gel	3[1]	8[2]
[1,2] Significant differences across columns, $P<0.01$ (Mann-Whitney U test)		
% Probes ≤50 µm	13 ± 4	11 ± 4
% Probes 50-100 µm	56 ± 4	54 ± 4
% Probes >100 µm	32 ± 4	36 ± 4

d) Apterous migrant 10 aphids/gel	Agarose	Agarose – sucrose
n	6	6
No. of probes/gel	101	122
No. nymphs/gel	6[1]	12[2]
[1,2] Significant differences across columns, $P<0.01$ (Mann-Whitney U test)		
% Probes ≤50 µm	12 ± 4	10 ± 4
% Probes 50-100 µm	46 ± 4	53 ± 4
% Probes >100 µm	42 ± 4	37 ± 4

6.3.1.2. Pymetrozine

A. craccivora was significantly deterred from probing on both types of artificial diets when they were mixed with pymetrozine. In contrast to the sachets, aphids produced significantly more offspring on pymetrozine treated gels.

Parafilm sachets with 100-1000ppm

Aphids probed significantly less on a 20% sucrose solution containing 100 to 1000ppm pymetrozine compared to aphids feeding on the control diets (20% sucrose) ($P<0.001$; Kruskal-Wallis) and the probes were significantly smaller (<100µm) ($P<0.05$; Logit Analysis); (Table 6.3a).

Parafilm sachets with 25-100ppm

The observed effects described for the higher concentrations of pymetrozine on aphid feeding behaviour were also noted at lower concentration (see previous section). The number of recorded probes on the pymetrozine containing diets was significantly less than on the control diets ($P<0.001$; Kruskal-Wallis); (Table 6.3b). The probes were not significantly smaller than on the control diets but due to the small number of probes, these statistics were not presented.

Agarose gels with 100-1000ppm

Aphids probed significantly less on agarose gels mixed with pymetrozine ($P<0.001$; Kruskal Wallis) and the probes were significantly shorter (<50µm), ($P<0.001$; Logit analysis) than the probes recorded on the control diets (Table 6.4a). However, irrespective of the pymetrozine concentration, significantly more nymphs were recorded on these gels compared to the control gels ($P<0.01$; Kruskal Wallis).

Agarose gels with 25-100ppm

Results obtained when agarose gels were mixed with lower concentrations of pymetrozine were similar to those described for the higher concentrations (see previous section). Significantly fewer and shorter probes were recorded on the pymetrozine containing diets while more offspring were produced ($P<0.01$ Kruskal Wallis), (Table 6.4b).

Table 6 3: Number and length of probes from 10 aphids confined on diets in parafilm sachets for 18h. Sachets contained pymetrozine ranging from 100 to 1000ppm (Table 6.3a) and 25 to 100ppm (Table 6.3b); n indicates number of replicates

a)

10 aphids/sachet		**Pymetrozine**		
	20% Sucrose	**100 ppm**	**300 ppm**	**1000 ppm**
n	6	6	6	6
No. of probes/sachet	76[1]	10[2]	8[2]	4[2]
No. of nymphs/sachet	4[1]	5[1]	7[1]	3[1]
[1,2] Significant differences across columns $P<0.001$ (Kruskal-Wallis)				
% Probes ≤50 µm	17 ± 2[a]	12 ± 5[a]	10 ± 6[a]	14 ± 9[a]
% Probes 50-100 µm	49 ± 3[a]	67 ± 7[b]	65 ± 7[b]	77 ± 10[b]
% Probes >100 µm	35 ± 3[a]	21 ± 6[b]	24 ± 7[b]	9 ± 8[b]
[a,b] Significant differences across columns $P<0.05$ (Logit Analysis)				

b)

10 aphids/sachet		**Pymetrozine**		
	20% Sucrose	**25 ppm**	**75 ppm**	**100 ppm**
N	3	3	3	3
No. of probes/sachet	47[1]	5[2]	3[2]	2[2]
No. of nymphs/sachet	8[1]	8[1]	6[1]	10[1]
[1,2] Significant differences across columns $P<0.001$ (Kruskal Wallis)				
% Probes ≤50 µm	37 ± 4	27 ± 13	33 ± 18	43 ± 19
% Probes 50-100 µm	53 ± 4	53 ± 13	56 ± 18	43 ± 19
% Probes >100 µm	10 ± 3	20 ± 14	0	0

Table 6 4: Number and length of probes from 10 aphids confined on diets as agarose gels (1.2% w/v) for 18h. Gels contained pymetrozine ranging from 100 to1000ppm (Table 6.4a) and 25-100ppm (Table 6.4b); n indicates number of replicates

a)

10 aphids/gel		**Pymetrozine**		
	Agarose	**100 ppm**	**300 ppm**	**1000 ppm**
N	6	6	6	6
No. of probes/gel	108^{1}	33^{2}	19^{2}	8^{2}
No. of nymphs/gel	16.5^{1}	68^{2}	52^{2}	74^{2}
^{1,2} Significant differences across columns $P<0.01$ (Kruskal Wallis)				
% Probes ≤50 µm	17 ± 2^{a}	39 ± 4^{b}	37 ± 5^{b}	36 ± 5^{b}
% Probes 50-100 µm	53 ± 2^{a}	45 ± 4^{b}	49 ± 5^{a}	47 ± 5^{a}
% Probes >100 µm	30 ± 2^{a}	17 ± 3^{b}	14 ± 4^{b}	16 ± 4^{b}
^{a,b} Significant differences across columns $P\leq0.001$ (Logit analysis)				

b)

10 aphids/gel		**Pymetrozine**		
	Agarose	**25 ppm**	**75 ppm**	**100 ppm**
N	3	3	3	3
No. of probes/gel	120^{1}	24^{2}	38^{2}	20^{2}
No. of nymphs/gel	12^{1}	36^{2}	49^{2}	60^{2}
^{1,2} Significant differences across columns $P<0.01$ (Kruskal Wallis)				
% Probes ≤50 µm	14 ± 2^{a}	46 ± 7^{b}	51 ± 6^{b}	50 ± 7^{b}
% Probes 50-100 µm	51 ± 3^{a}	41 ± 6^{a}	44 ± 6^{a}	43 ± 6^{a}
% Probes >100 µm	35 ± 3^{a}	13 ± 4^{b}	6 ± 4^{b}	7 ± 4^{b}
^{a,b} Significant differences across columns $P\leq0.001$ (Logit analysis)				

6.3.1.3. Neem-oil

Ethanol extracts of neem-oil showed feeding deterrency to *A. craccivora* in both bioassays, but the deterrent effects were different than those observed for pymetrozine (6.3.2.2). Significantly fewer were recorded on neem-based diets either in sachets (Table 6.5) or agarose gels (Table 6.6), but probes were also significantly longer. Ethanol did not have an effect on aphid feeding.

Table 6 5: Number and length of probes from 10 aphids confined on diets in parafilm sachets for 18h. Sachets contained 0.022% neem and 0.05%EtOH in a sucrose 20% solution or 0.05% EtOH in 20% sucrose only (control). Number of replicates, n=5.

10 aphids/sachet	20% Sucrose	20% Sucrose (0.05%EtOH)	Neem 0.022% (0.05%EtOH)
N	3	5	5
No. of probes/sachet	99[1]	100[1]	29[2]
No. of nymphs/sachet	9[1]	10[1]	11[1]
[1,2] Significant differences across columns $P<0.001$ (Kruskal-Wallis)			
% Probes ≤50 µm	33 ± 3[a]	40 ± 2[a]	13 ± 3[b]
% Probes 50-100 µm	60 ± 4[a]	54 ± 3[a]	50 ± 5[a]
% Probes >100 µm	7 ± 2[a]	6 ± 1[a]	37 ± 6[b]
[3,4] Significant differences across columns $P<0.01$ (Logit Analysis)			

Table 6 6: Number and length of probes from 10 aphids confined on diets as agarose gels (1.2% w/v) for 18h. The gels contained 0.022% neem in 0.05% EtOH or 0.05% EtOH only (control). Number of replicates, n=7.

10 aphids/gel	Control gels	Gels with 0.022% neem in 0.05% EtOH
N	7	7
No. of probes/gel	85[1]	35[2]
No. of nymphs/gel	13[1]	11[1]
[1,2] Significant differences across columns $P<0.001$ (Mann-Whitney U test)		
% Probes ≤50 µm	18 ± 2[a]	17 ± 3[a]
% Probes 50-100 µm	62 ± 2[a]	42 ± 4[b]
% Probes >100 µm	20 ± 2[a]	41 ± 4[b]
[a,b] Significant differences across columns $P\leq0.001$ (Logit analysis)		

6.3.2. The effect of plant extracts of groundnut in artificial diets on the feeding behaviour of *A. craccivora*

6.3.2.1. Extracts of leaf tissue

Aphids probed more and produced longer probes on water extracts of leaf tissue either applied in parafilm sachets or agarose gels. The differences were significant for aphids feeding on the sachets but not on the gels.

Parafilm sachets

Significantly more probes were recorded on extract-containing diets than on the water control diets but not the sucrose containing diets ($P<0.01$, Kruskal Wallis) (Table 6.7). However, the probes were also significantly longer than on both control diets indicating probing stimulatory effects ($P\leq0.01$, Logit analysis). Differences between the extracts of JL24 and ICG12991 on the feeding behaviour of *A. craccivora* were not identified. Similar results on aphid feeding behaviour on 10-times diluted extracts were observed assuring that a feeding effect on agarose gels containing the leaf extracts could potentially be detected.

Agarose gels

Although aphids probed more on extract-containing gels, this was not significant ($P=0.1$, Kruskal Wallis), (Table 6.8). The probes were, however, significantly longer on the extracts of either resistant and susceptible variety than on the control diets and also probes were longer on extracts of ICG12991 than on those of JL24 (>100µm; $P<0.01$, Logit analysis).

Table 6 7: Number and length of probes from 5 aphids confined on diets in parafilm sachets for 18h. Sachets contained water extracts of leaf tissue (1g/5ml) of either a susceptible (JL24) or resistant (ICG12991) groundnut variety (n=10), 20% sucrose or distilled water only (control) (n=5).

5 aphids/sachet	Distilled water	20% Sucrose	Leaf extract of JL24	Leaf extract of ICG12991
n	5	5	10	10
No. of probes/sachet	9^{1}	43^{2}	33^{2}	30^{2}
^{1,2} Significant differences across columns $P<0.01$ (Kruskal Wallis)				
% Probes ≤50 µm	74 ± 6^{a}	42 ± 3^{b}	32 ± 4^{c}	29 ± 4^{c}
% Probes 50-100 µm	21 ± 7^{a}	45 ± 3^{b}	44 ± 3^{c}	42 ± 4^{c}
% Probes >100 µm	4 ± 4^{a}	13 ± 2^{a}	24 ± 4^{b}	30 ± 5^{b}
^{a,b,c} Significant differences across columns $P<0.01$ (Logit analysis)				

Table 6 8: Number and length of probes from 5 aphids confined on diets as agarose gels (1.2% w/v) for 18h. The gels contained water extracts of leaf tissue (1g/5ml) of either a susceptible (JL24) or resistant (ICG12991) groundnut variety (n=8).

5 aphids/gel	Agarose (Control)	Leaf extract of JL24	Leaf extract of ICG12991
N	7	8	8
No. of probes/gel	123	172	191
P=0.1, Kruskal Wallis			
% Probes ≤50 µm	16 ± 2^{a}	11 ± 2^{b}	9 ± 1^{b}
% Probes 50-100 µm	47 ± 2^{a}	$46 \pm 3^{a,b}$	40 ± 2^{b}
% Probes >100 µm	36 ± 2^{a}	43 ± 2^{b}	51 ± 3^{c}
^{a,b,c} Significant differences across columns $P<0.01$ (Logit analysis)			

6.3.2.2. Diluted extracts of leaf tissue

A more detailed comparison of aphid feeding behaviour on sachets containing diluted extracts of either extracts or JL24 or ICG12991 did not reveal clear differences. Leaf extracts at the concentration of 1mg dry-weight in 1ml distilled water did not stimulate feeding in terms of number of probes, when compared to 20% sucrose and distilled water, although probes were longer (Table 6.9a). On the diluted extracts between varieties, aphids behaved similarly and both number of probes and length of probes were longer than on the control diets (Table 6.9b-d).

Table 6 9: Number and length of probes from 5 aphids confined on diets in parafilm sachets for 18h: a) Sachets contained water extracts of leaf tissue (1mg/1ml) of either a susceptible (JL24) or resistant (ICG12991) groundnut variety (n=7), b) 10X diluted extracts; c) 100X diluted and c) 100X diluted. Controls were 20% sucrose or distilled water only (control) (n=2).

a) 5 aphids/sachet	Distilled water	20% Sucrose	1mg/1ml Leaf extract of JL24	1mg/1ml Leaf extract of ICG12991
N	2	2	7	7
No. of probes/sachet	6	15	6	6
% Probes ≤50 µm	75	57	32	41
% Probes 50-100 µm	17	40	54	54
% Probes >100 µm	7	3	14	5

b) 5 aphids/sachet	Distilled water	20% Sucrose	Dilution 10X Leaf extract of JL24	Dilution 10X Leaf extract of ICG12991
N	2	2	7	7
No. of probes /sachet	4	11	22	32
% Probes ≤50 µm	92	54	26	23
% Probes 50-100 µm	8	42	41	40
% Probes >100 µm	0	4	33	37

c) 5 aphids/sachet	Distilled. water	20% Sucrose	Dilution 100X Leaf extract of JL24	Dilution 100X Leaf extract of ICG12991
N	2	2	7	7
No. of probes/sachet	5	32	38	37
% Probes ≤50 µm	58	24	29	34
% Probes 50-100 µm	25	57	42	47
% Probes >100 µm	17	19	29	19

d) 5 aphids/sachet	Distilled. water	20% Sucrose	Dilution 1000X Leaf extract of JL24	Dilution 1000X Leaf extract of ICG12991
N	2	2	7	7
No. of probes/sachet	4	18	22	17
% Probes ≤50 µm	76	47	30	35
% Probes 50-100 µm	18	44	33	39
% Probes >100 µm	6	9	37	26

6.3.2.3. Extracts of flower tissue

Diets containing water extracts of flower stems (0.3g/5ml) from a susceptible (JL24) and resistant (ICG12991) groundnut variety stimulated aphid feeding significantly. More and longer probes were recorded on the extract-containing diets than on the control diets. The differences in aphid feeding behaviour were observed in both bioassays and aphid feeding did not differ on extracts of either JL24 or ICG12991.

Parafilm sachets

Aphids probed significantly more on the extract-containing diets than on water control and the sucrose solutions (Table 6.10). The recorded probes were also significantly longer ($P<0.01$, Logit analysis). Aphids also made significantly fewer smaller probes on sachets containing extracts of ICG12991 than those containing extracts of JL24 ($P<0.01$, Logit analysis).

Similar results were obtained on 10-times diluted extracts (data not shown) and a similar observation may therefore be detected on agarose gels. Similar results on aphid feeding behaviour on 10-times diluted extracts were observed assuring that a feeding effect on agarose gels containing the leaf extracts could potentially be detected.

Agarose gels

On extract–containing gels of either susceptible and resistant variety, aphids probed significantly more and longer than on control diets (Table 6.11). Additionally, aphid-probes were significantly longer on gels containing the flower extracts of the resistant ICG12991 than those containing the extracts of JL24. The result was similar as observed for aphids feeding on leaf extracts of both varieties (6.3.3.2).

Table 6 10: Number and length of probes from 5 aphids confined on diets in parafilm sachets for 18h. Sachets contained water extracts of flower stems of either a susceptible (JL24) or resistant (ICG12991) groundnut variety (n=9), 20% sucrose (n=9) or distilled water (control, n=8). Number of replicates, n=9.

5 aphids/sachet	Distilled water	Sucrose 20%	Extract of flower of JL24	Extract of flower of ICG12991
N	8	9	9	9
No. of probes/sachet	10^{1}	20^{2}	57^{3}	38^{3}
[1,2,3] Significant differences across columns $P<0.05$ (Kruskal Wallis)				
% Probes ≤50 µm	80 ± 5^{a}	49 ± 3^{b}	46 ± 3^{b}	33 ± 3^{c}
% Probes 50-100 µm	14 ± 5^{a}	49 ± 3^{b}	39 ± 4^{c}	$44 \pm 3^{b,c}$
% Probes >100 µm	6 ± 3^{a}	7 ± 1^{a}	21 ± 4^{b}	23 ± 4^{b}
[a,b,c] Significant differences across columns on $P\leq0.01$ (Logit analysis)				

Table 6 11: Number and length of probes from 5 aphids confined on diets as agarose gels (1.2% w/v) for 18h. The gels contained water extracts of flower stems of either a susceptible (JL24) or resistant (ICG12991) groundnut variety. n=9.

5 aphids/sachet	Agarose (Control)	Extract of flower of JL24	Extract of flower of ICG12991
N	8	9	9
No. of probes/gel	62^{1}	148^{2}	178^{2}
[1,2] Significant differences across columns $P<0.001$ (Kruskal Wallis)			
% Probes ≤50 µm	23 ± 2^{a}	14 ± 2^{b}	11 ± 2^{b}
% Probes 50-100 µm	43 ± 2^{a}	43 ± 3^{a}	39 ± 3^{a}
% Probes >100 µm	35 ± 2^{a}	44 ± 3^{b}	50 ± 3^{c}
[3,4,5] Significant differences across columns $P\leq0.001$ (Logit analysis)			

6.3.4. Summary table on the effects of probing stimulants, deterrents and plant extracts on aphid feeding behaviour on two bioassays

Feeding deterrency was always characterised by fewer probes per sachet or gel (Pymetrozine, neem-oil extract) and this was not observed after aphids had fed on extracts of groundnut varieties. In contrast, feeding stimulatory effects were always characterised by more and longer probes in sachets and gels. This was always observed after aphids had fed on groundnut extractions of either a susceptible (JL24) or resistant variety (ICG12991) (Table 6.12).

Table 6 12: Observation of compounds on feeding behaviour of *A. craccivora* as measured by number (No.) and length of probes recorded on diets either in parafilm sachets or agarose gels. The symbols + and – indicate a stimulatory and deterrent effect respectively as compared to a control (sucrose 20% in sachets and agarose 1.2% w/v in gels) in a subgrading system according to results obtained and presented in the tables (6.1-6.11). Sucrose 20% is in itself compared to water. The symbol " indicates a similar response of aphids feeding on the water control.

Compounds in the diets	**Artificial feeding system**							
	Parafilm Sachets				**Agarose gels**			
	No. of probes/sachet		Probe-length		No. of probes/gel		Probe-length	
20% Sucrose	+		+		"		"	
Pymetrozine	- - -		-		- - -		- -	
Neem-oil Extract	- - -		+ +		- - -		+ +	
Water extract of plant tissue of groundnut variety								
	JL24	ICG1291	JL24	ICG1291	JL24	ICG1291	JL24	ICG1291
	No. probes		Probe-length		No. probes		Probe-length	
Leaf extracts	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Flower extracts	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +

+'s and –'s denote size of the effects compared to the controls

6.4. Discussion

6.4.1. *A. craccivora* can detect compounds within agarose gels

The feeding experiments showed that *A. craccivora* could detect compounds within an agarose probing substrate which influenced its feeding behaviour. The recorded effects of the compounds were in line with those observed when aphids had fed on parafilm sachets, a technique developed to examine the effect of plant substances and other compounds on aphid feeding (Mittler & Dadd, 1962). In those experiments, aphid feeding was usually examined by honeydew excretion analysis but not by examining aphid probes. It was now observed that a feeding stimulatory effect was reflected in more and longer probes in the diets, while a deterrent effect was reflected in less but not necessarily smaller probes. Despite standardisation of aphid rearing and experimental set-up, the variation in the measurements taken stimulatory or deterrent effects relative to the controls. On gels and parafilm sachets containing plant extracts of a resistant (ICG12991) and susceptible variety (JL24), aphids probed to such an extent compared to the control diets that it was not likely that soluble chemicals could be associated with resistance in ICG12991. The results here supported the hypothesis that aphid resistance in ICG12991 was not constitutive but induced by aphid probing (Chapter 5).

6.4.2. How are the compounds perceived?

So far there was only evidence of stylet pathway activity of feeding aphids on agarose gels, which was measured using the electrical penetration graph technique (Urbanska *et al.*, 1998). The graphs corresponded to those from aphids feeding on epidermal and mesophyll tissues of plants and salivary sheath formation but were not conclusive on the presence of watery saliva within the gels. However, if aphids can detect compounds within agarose gels which could influence their behaviour, it would imply that apart from producing gelling saliva for stylet sheath formation, also watery saliva is ejected and ingested to taste the chemicals trapped in the gel. Aphids responded to gels containing pymetrozine, neem-oil and leaf and flower extracts by alteration of probing behaviour through variations in the number and length of probes. Aphid saliva may contain enzymes to break down the galactose linkages (Agarose is galactan or galactose

polymer) because galactose is a general sugar in plants. In the gels, two chains form a double helix in which water and its soluble components are enclosed and breakdown of the linkages may release substrates which can be actively sucked in and tasted with the gustatory organ in the pharynx. It is not conclusive whether the aphid saliva contains such enzymes but a similar theory could be applied to the results obtained by Staniland (1924). He impregnated a mixture of gelatine and glycerine with starch and added a little apple juice. The wooly aphid (*Eriosoma lanigerum,* Hausmann) fed on this stiff jelly and patches were visible where starch was converted into sugar. At the sites of these patches, minute quantities may be ingested although there is no evidence whether the patches were caused by some of the aphid's gelling saliva diffusing into the substrates or whether they indicate the presence of watery saliva and ingestion. The experiments with the wooly aphid on starch media could be repeated and analysed using number of patches and size of patches as parameters to analyse feeding behaviour, but were outside the objectives of this work. It was concluded that agarose gels mimic the tissues surrounding the sieve element as proposed by Urbansky and co-workers in 1998 and that aphids would be able to detect plant substances during pathway activity from intercellular spaces.

Sucrose, for example, is abundant in plants and may be found in high concentrations in the phloem as well as in the apoplast and vacuoles (Akazawa & Okamoto, 1980). The sucrose concentration in the apoplast for example may be sampled during intercellular pathway activity and may act as a probing and parturition stimulant. More nymphs were consistently recorded on agarose-sucrose gels, compared to the control gels, while more probes were recorded on sachets containing sucrose than on the control sachets containing water only. The different effects of sucrose on aphid behaviour observed in the different bioassays might give an indication of how the bioassays may complement each other. Prior to phloem location during probing, parturition stimulants are thought to be detected by aphids and probably other phloem-sucking insects (Powell & Hardie, 2001). Sucrose could be one such parturition stimulant and affect nymphal production as observed in gels but also stimulate feeding as observed in the sachets. The function of sucrose as a dietary one or not is open for debate and would require more experiments measuring feeding and reproduction and analysis of aphid salivary

enzymes. The feeding experiments on agarose gels only show the potential of the bioassay to detect the effect plant substances can have on aphid behaviour during stylet pathway activity. The scale of the stimulatory effect of aphids feeding on gels containing leaf and flower extract compared to the stimulatory effect of sucrose only emphasized this potential.

6.4.3. Interpretation difficulties of feeding parameters in diets

Interpretation of the results may not always be obvious. Stimulatory effects were readily recognized by an increase in number and length of probes occurring simultaneously but deterrent effects varied between pymetrozine and neem-oil.

On pymetrozine containing diets, the feeding deterrent effect in both bioassays was reflected in fewer and smaller probes but the number of nymphs recorded on the pymetrozine containing diets was very high. A plausible explanation could lie in the way pymetrozine acts against aphid feeding. Aphids were deterred from probing on diets containing pymetrozine and on ethanol extracts of neem-oil. Pymetrozine causes the aphid to stop feeding, which is not resumed and therefore aphids starve. It is assumed that pymetrozine blocks aphid feeding by interfering with the control system in the brain and is active via both ingestion and contact. The inability to feed again may have subsequently increase nymphal production to secure survivors of the next generation. The efficacy of pymetrozine in pest control has already been demonstrated against *Aphis gossypii* Glover, *Myzus persicae* Sulz. , *Macrosiphum euphoribiae* Thomas, *Bemisia* spp. and *Trialeurodes vaporariorum* Westwood on several field crops (Allemann *et al.*, 1994; Flueckiger *et al.*, 1992a,b; Nicholson *et al.*, 1996) including those in the tropics (Senn *et al.*, 1994).

In contrast to pymetrozine, aphids produced fewer but longer probes on diets containing ethanol extracts of neem-oil, a known aphid anti-feedant (Butterworth & Morgan, 1968) compared to the control. They were also more robust, which could be due to the absorbance of the chemical in the stylet sheath, one of the functions originally allocated to the stylet sheath to overcome plant defence systems (Miles, 1999). Why the probes were longer is not clear but it could be related to an increased searching behaviour for xylem vessels. It is known that "drinking" from xylem in the plants, helps the insect to maintain a sufficiently large water turnover for removal of toxicants (Stobbart & Shaw,

1974). In related research, body shrinkage was reported in 5th-instar rice earcutting caterpillar (*Mythimna separata*), which had been feeding on rice leaves dipped in a methanolic neem seed extract for 24h (Schmutterer *et al.*, 1983). This shrinkage was due to cessation of feeding and increased excretion. The effect of the neem-extract, could be ascribed to ingestion from the artificial diets rather than via contact. From the literature it could be noted that honeydew production by *Myzus persicae* was reduced by more than 80% when 500-1000ppm of azadirachtin was incorporated into a complex artificial diet indicating post ingestion deterrence (Nisbet *et al.*, 1994).

EPG recording of feeding aphids on both types of diets would be a helpful tool to better understand their behavioural response to the feeding deterrent and stimulant chemicals. Ingestion from the diets was not measured and therefore all the results can only be related to aphid probing behaviour. As it was established that aphid resistance in groundnut variety ICG12991 was perceived prior to salivation into the phloem sieve elements (Chapter 5), these experiments here have shown that there is no evidence that a constitutive chemical associated with ICG12991 was responsible for the aphid resistance. The hypothesis that aphid probing may induce physiological changes which may or may not result in the expression of a hypersensitive response and that these changes may be detected in the apoplastic fluids during stylet pathway activity gained more credibility. As a result stylets are withdrawn and the plants rejected. In the following chapter, the stability of this proposed mechanism is examined.

CHAPTER 7

DURABILITY OF APHID RESISTANCE IN GROUNDNUT VARIETY ICG12991

7.1. Introduction

Over the course of their evolution, aphids have evolved many unique and effective means with which to utilise their host plant for food and shelter (Dixon, 1989). The ability to avoid vacuole-sequestered toxins by moving their stylets intercellularly, combined with parthenogenetic reproduction has made this group one of the most successful groups of phytophagous insects. The success though has also made them one of the most devastating group of pests in crops world-wide, reducing yields either directly by removing enough photosynthates to stunt plant growth or indirectly mainly as vectors for pathogens. (Rabbinge *et al.*, 1981). Because the Aphididae transmit more than 275 viruses (Nault, 1997), introducing vector resistant varieties to control the viruses they transmit should be an important strategy to control aphid vectored virus diseases (Jones, 1998; Chapter 4). Successful releases of vector resistant material to control non-persistent viruses such as plum pox virus in Europe (Maison & Massonie, 1982); semi-persistent viruses such as aphid transmitted viruses in raspberry from Britain and America (Jones, 1976) and persistent viruses such as *Potato leafroll virus* in several solanum species (Radcliffe & Lauer, 1970) were reported. However, resistant breaking aphid biotypes, following the release of resistant varieties have also been described (Atiri *et al.,* 1984; Dahal *et al.,* 1990; Birch *et al.,* 1992, 1994). Vector resistance to *Groundnut rosette virus* disease in selected groundnut varieties, such as ICG12991, and its significance in virus transmission has been well documented (Chapters 2, 3, 4 and 5) but the release of this variety in key areas of Africa to control the disease will only be effective if the resistance is durable. Because the planting and promoting of vector resistant varieties is becoming a significant part of an integrated management strategy for groundnut rosette disease in sub-Saharan Africa (Chancellor,

2002), the probability of *A. craccivora* to develop into a resistant breaking biotype and to become a more efficient vector when large areas of aphid resistant varieties are planted over time, should be considered.

The biotype concept is a controversial one. It was initially introduced to describe and differentiate the variation that occurs in insect populations (Srivastava & Auclair, 1990). Aphid-biotypes were initially considered to be populations of genetically identical individuals that reproduce parthenogenetically and differ in their abilities to develop and survive on various plant species within their host range (review by Swenson, 1968), which in this context only referred to interspecific variation. Then, in 1973 biotypes were characterized on the basis of differential host plant utilisation within a species (Eastop, 1973), which in this context referred to intraspecific variation. Some researchers have even chosen other criteria than differential host plant utilisation to characterise biotypes, such as insecticide resistance, which have caused overlaps in biotype designations within an insect species. As a result, several biotypes may be separated by one trait, but grouped together when considering another trait (Emden van *et al.*, 1969) and this inconsistency has confused the biotype concept to the point where its usage is regarded as having no distinct biological meaning (Claridge & Den Hollander, 1983) or as an ambiguous term that should be abandoned (Diehl & Bush, 1984). Despite this controversy the concept is still widely used and numerous studies on aphids and whiteflies have shown that different biotypes and populations show differences in virus transmission efficiency (Bedford *et al*, 1994), performance on different host-plant species (Bethke *et al.*, 1991), ability to produce phytotoxic-like disorders (Shapiro, 1996), propensity to migrate (Byrne & Blackmer, 1996), and resistance to insecticides (Denholm *et al.*, 1996).

Aphid performance, feeding behaviour and transmission efficiency were investigated to examine whether long-term rearing of *A. craccivora* on the resistant groundnut variety ICG12991 would result in aphid populations that could overcome the resistance in this variety.

7.2. Materials & Methods

7.2.1. Population establishment of *A. craccivora* on ICG12991

To establish aphid colonies on the resistant groundnut variety ICG12991, young plants (14 DAP) were manually infested with 10 adult apterous aphids. The adults were removed after 48h and although a relatively high mortality among nymphs was likely (Chapter 2), a population was readily established. Groundnut plants were regularly replaced by fresh young plants (14 DAP). Prior to the experiments 10-15 adult aphids were used to start a new colony and newly moulted adults of similar age were selected.

7.2.1.1. Adaptation to new host plants of same species

A population of *A. craccivora* was maintained on the aphid resistant variety ICG12991 for approximately 1.5 years as described in 7.2.1. Aphids from this population will be referred to as 'adapted aphids' (AA) as opposed to aphids reared on susceptible plants (var. Malimba) since 1997, which will be referred to as the 'susceptible aphids' (SA). One adult apterous aphid from each culture (SA + AA) was placed on separate plants (10 DAP) of JL24 (susceptible control), ICG12991 and ICG SM99540 (aphid resistant) for 24h to produce first instar nymphs (Chapter 2). The adults were then removed and 5 nymphs per plant were allowed to develop into adulthood. When reaching adulthood, one aphid was carefully moved to a new 10 DAP plant of the same variety to generate new offspring (2^{nd} generation aphids) in the following 24h. The remaining 4 aphids were quickly frozen at –80°C and weighed on a Mettler AT201 balance (sensitivity of 0.01mg). When 5 nymphs of the second generation reached adulthood, the process was repeated until 6 generations were followed up. Five plants per variety were used so that 20 adults per generation per variety were weighed. Data from the fourth generation were missing.

7.2.1.2. Aphid performance of an established and non-established colony on ICG12991

Performance of AA and SA (n=10) on ICG12991 and JL24 was estimated by the intrinsic rate of increase (Rm) (Wyatt & White, 1977). Test plants were 21 DAP and the experimental design was identical to the one described in Chapter 2. The plant-age was selected because data had already been gathered on aphid performance on 7 DAP and 28 DAP plants (Chapter 2).

7.2.1.3. Feeding behaviour of adapted and non-adapted aphids on ICG12991

Honeydew deposits were collected from an ICG12991 adapted (AA) and non-adapted aphid population (SA) on ICG12991. Five adult apterous aphids from either population were placed in a clip-cage containing a 'water and oil sensitive' paper (TeeJet, Spraying Systems Co®; Chapter 5) and honeydew was collected over 24h feeding period. Plants were 10 DAP (n=8) and 28 DAP (n=16). The number of deposits was counted after 24h and the diameter of the deposits measured under a binocular microscope, magnification (10X12). The HD-size was classified as ≤0.5mm or >0.5mm indicating it was excreted by a nymph or an adult respectively.

7.2.2. Transmission efficiency of the virus agents by aphids that were reared long-term on a resistant groundnut variety

Viruliferous aphids and susceptible source plants (var. Malimba), containing all three virus agents were maintained as described in Chapter 4.

Five adult apterous viruliferous aphids from both populations (SA + AA) were placed on ICG12991 and JL24 (7 DAP) for an inoculation access period (IAP) of 48h. Two weeks later the plants were tested for the virus agents based on symptoms (GRV + satRNA) and RT-PCR (detection of GRAV). Only one block of five plants per treatment was conducted.

7.3. Results

7.3.1. Population establishment of *A. craccivora* on ICG12991

7.3.1.1. Adaptation to new host plants of same species

Aphids which were continuously reared on ICG12991 (AA) weighed approximately 0.2mg at the start of the experiment but their weight recovered within 3 generations when placed on the susceptible JL24 to the level of aphids reared continuously on susceptible varieties to approximately 1mg (Figure 7.1a, also see Chapter 2). When new generation aphids were transferred to young, healthy plants of ICG12991, their weight (AA) gradually increased until equilibrium was reached of approximately 0.5mg (Figure 7.1b). The same trend was observed when they were transferred to ICG SM99540 (Figure 7.1c).

In contrast, aphids from the susceptible population (SA) showed the opposite trend and their weight decreased over 3 generations when they were developing on the resistant varieties ICG12991 and ICG SM99540 but it stabilised at approximately 0.5mg (Figure 7.1b-c).

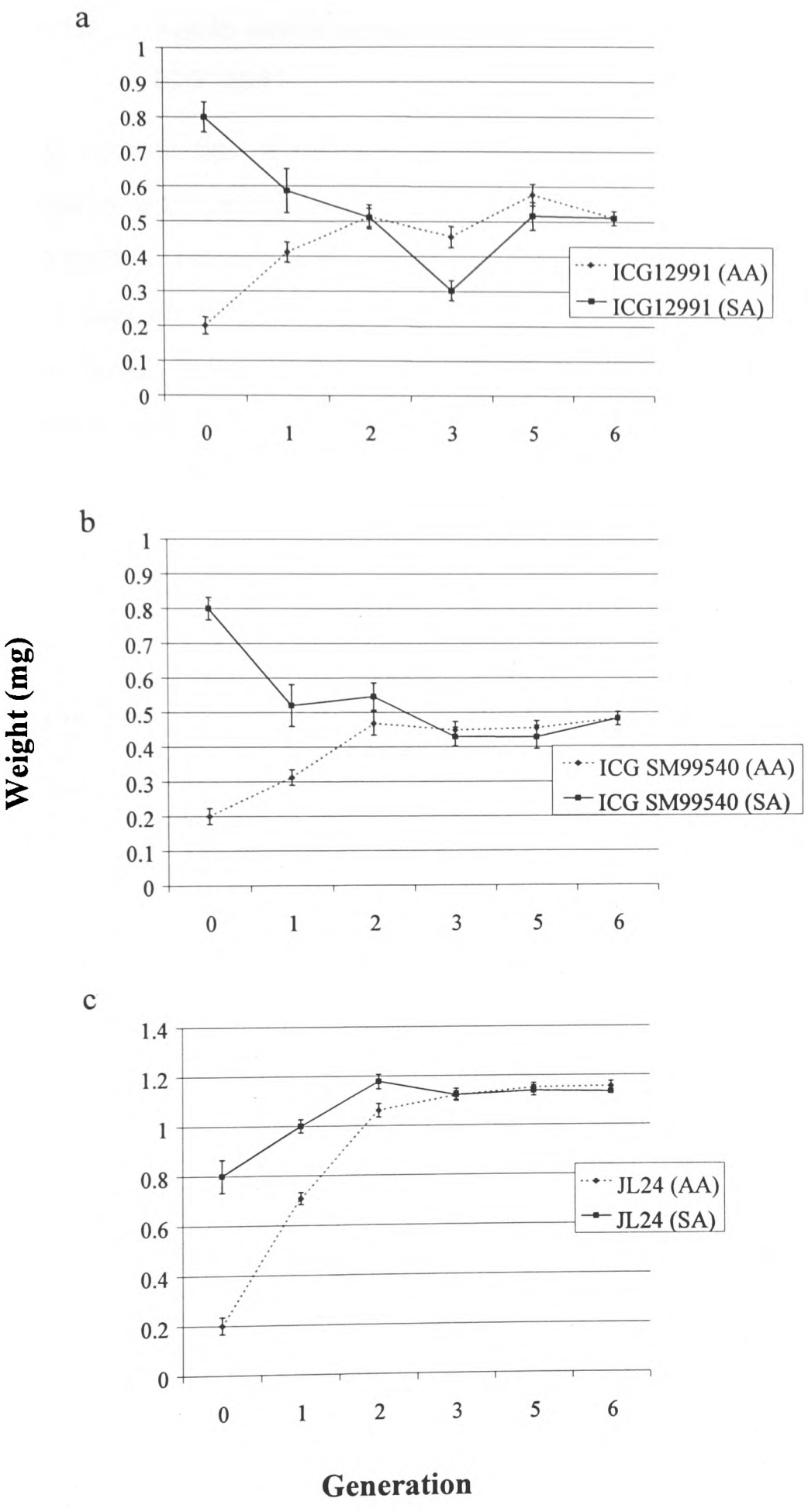


Figure 7 1: Mean aphid weight (±S.E.) at adulthood over 6 generations on three groundnut varieties. Aphids which were continuously reared on susceptible varieties (var. Malimba), are represented by SA, while those aphids which were continuously reared on resistant variety ICG12991, are represented by AA. Aphids were transferred to young test plants of each variety of 10 DAP. 25 aphids per generation were weighted. Data on generation 4 are missing.

7.3.1.2. Aphid performance of an established and non-established colony on ICG12991

The intrinsic rate of increase (Rm) for aphids on 21 DAP plants of ICG12991 were based on a limited amount of data points due to high mortality of both SA (70%) and AA (50%). The surviving nymphs of AA and SA developed into the reproductive stage in 7 days on ICG12991 and only 6 days on JL24 (Table 7.1). Aphid fecundity was significantly lower for both SA (Md=17) and AA (Md=17) on ICG12991, compared to their fecundity when feeding on JL24 (Md=58 and 40 respectively). Due to the low number of replicates, statistics were not applied.

Table 7 1: The intrinsic rate of increase (Rm) of aphids on a resistant (ICG12991) and susceptible groundnut variety (JL24). Plants were 21 DAP (days after planting) and the aphids were collected from a population established on the same varieties, JL24 (SA) and ICG12991 (AA). D= aphid development time in days; Md= fecundity in time D; n= number of replicates.

Aphid population	Variety	Aphid performance parameters		
		D	Md	Rm
SA	JL24	5.6 ± 0.2 n=9	58 ± 2 n=9	0.54 ± 0.02 n=9
	ICG12991	6.7 ± 0.2 n=6	17 ± 3 n=3	0.31 ± 0.03 n=3
AA	JL24	5.8 ± 0.2 n=9	41 ± 1 n=9	0.48 ± 0.01 n=9
	ICG12991	6.9 ± 0.3 n=7	17 ± 2 n=5	0.30 ± 0.03 n=5

7.3.1.3. Feeding behaviour of adapted and non-adapted aphids on ICG12991

Aphids from the adapted population (AA) produced significantly more but smaller honeydew deposits (HD) on ICG12991 than the non-adapted aphid population (SA) ($P<0.001$; Mann-Whitney *U* test) (Figure 7.2, Table 7.2). The mean number of nymphs recorded after 24h was similar for both SA and AA but nymphs from AA also excreted more HD ($P<0.001$; Mann-Whitney *U* test), stressing the adaptation of aphids when confined on ICG12991.

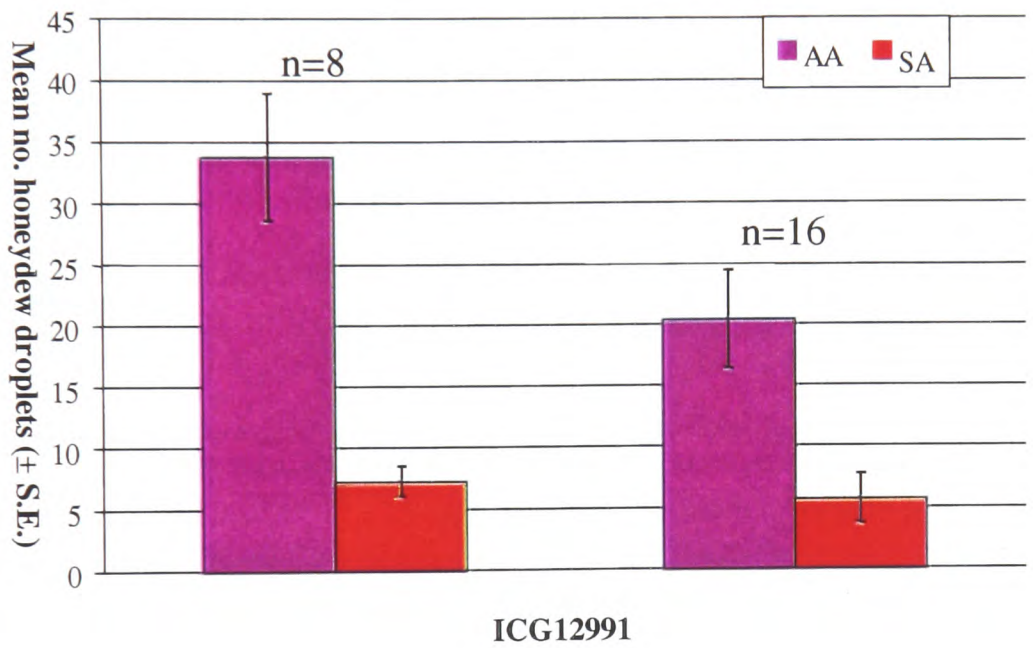


Figure 7 2: Mean number of honeydew deposits collected after 24h when 5 adult apterous aphids were confined in clip-cage on plants of ICG12991. Number of replicates, n=8 (10 DAP) and n=16 (28 DAP).

Table 7 2: Total number of honeydew deposits (HD) excreted by aphids from an ICG12991 adapted (AA) and non-adapted (SA) population. Five adult apterous aphids per replicate (n=16) were confined in clip-cages on ICG12991 (28 DAP) for 24h. The proportions of nympal- and adult HD were calculated

Aphid population	Total no. of HD	Mean no. of nymphs ± S.E.	Total no. HD excreted by nymphs< 0.5mm	Total no, of HD excreted by adults > 0.5mm
SA	91	5 ± 0.7	60 (66%)	31 (34%)
AA	327	5 ± 0.7	140 (43%)	187 (57%)

7.3.2. Virus transmission efficiency of aphids adapted to the resistant variety ICG12991

Aphids that were adapted to ICG12991 (AA) did not transmit the disease agents more efficiently than those not adapted (SA) (Table 7.3). SA inoculated 60% of the plants of ICG12991 with at least one viral agent and 20% of the plants were inoculated with all three agents. AA inoculated 80% of the plants with at least one agent and also 20% of the plants were inoculated with all three agents. On the susceptible control JL24 a 100% infection occurred and 80% of the plants were inoculated with all three agents for both aphid clones.

Table 7 3: Transmission of GRD agents by aphids adapted (AA) and not adapted to ICG12991 (SA). Number of replicates n=5 for each treatment

Aphid population and transmission of virus agents on 7DAP plants	**5 viruliferous aphids per plant on groundnut variety**			
	ICG12991 (n=5)		**JL24 (n=5)**	
Virus agents	**Aphid population**		**Aphid population**	
	SA	**AA**	**SA**	**AA**
All three agents	1	1	4	4
GRV + satRNA	0	0	0	1
GRAV	2	3	1	0
No agents	2	1	0	0

7.4. Discussion

The population of *A. craccivora* originally collected from Soroti, Uganda, adapted within 3 generations on resistant groundnut varieties ICG12991 and ICG SM99540 under laboratory conditions. This was achieved as long as seedlings were used and replaced every 10 days. A population was easily established on ICG12991 but high mortality rates were observed when the adapted aphids were placed on older plants of ICG12991 (21 DAP). However, the adaptation of aphids on ICG12991 was not without a cost even on very young plants. Aphids on ICG12991 were less fit than those on JL24 and this was reflected in their reduced reproduction and bodyweight. However, the effect was reversible and within another 3 generations on a susceptible host, the aphids had a similar weight to the control group. The reduced fecundity and loss of bodyweight of adapted aphids on ICG12991 resulted presumably because they were unable to feed on this variety. This is supported by the observations of honeydew excretion, which showed that adapted aphids on ICG12991 produced fewer deposits compared to those produced by aphids on JL24. The adapted aphids were likely to have periods of sustained phloem feeding on ICG12991, which was visualised by the excretion of more honeydew deposits compared to those excreted by non-adapted aphids. Also adapted first instar nymphs excreted more honeydew deposits than those from the non-adapted population but it appeared that their feeding was interrupted. In the field it could be suggested that the smaller size of the aphids on ICG12991 would make them more vulnerable to external factors (Dixon, 1985a), although given the interrupted feeding behaviour it is debatable whether individuals of *A. craccivora* would remain on the plants long enough to be able to adapt on this variety or not. In these conditions difficulties in establishing sustained feeding could result in dehydration and death.

The hypersensitive response (HR), as outlined in Chapter 5, was still expressed around the feeding sites of adapted aphids. Therefore it is suggested that even if *A. craccivora* can survive on plants of ICG 12991, they may be adapted to the increased oxidative status of the plant but cannot overcome cell death associated with hypersensitivity. The HR may have caused the aphids to withdraw their stylets and the reduced size and weight may simply compensate for the limited phloem uptake on ICG12991, but this

needs to be tested further. The electronic monitoring system (EPG) is needed to fully characterise the feeding behaviour of the adapted aphids. However, as was discussed in Chapter 5, aphid feeding and the induction of the HR are linked and have consequences for virus transmission efficiency. Aphids that were adapted to ICG12991 were not more efficient in transmitting any virus agent of groundnut rosette disease than non-adapted aphids. If the virus complex is inoculated into plant cells which die as a result of the HR then the viruses would not be able to replicate and move from the site of infection into neighbouring cells.

In summary, the results indicated that under laboratory conditions the aphid population collected in Uganda was able to adapt to the variety ICG12991 within three generations but the effect was reversible within another three generations. Adapted aphids were not however, able to infect ICG 12991 with the rosette virus complex. As mentioned in the introduction the biotype concept is debated widely, but according to all the definitions given, the reversible effect noted in these experiments suggest that the adapted population of *A. craccivora* does not represent a new biotype. The populations which were found on weed species and the cowpea plants in Uganda and Malawi, however, may represent distinct biotypes but this would need to be confirmed through a separate series of trials and possibly molecular analyses as used in the molecular typing of whiteflies (Perring *et al.*, 1993).

CHAPTER 8

GENERAL DISCUSSION

The work in this study has focused on the evaluation and elucidation of the resistance mechanism in ICG12991 because it is a high yielding variety with desirable characteristics which meet market requirements in southern and eastern Africa (P.J.A. van der Merwe, pers.comm). ICG12991 was totally susceptible to the virus as was shown in grafting experiments (Merwe van der *et al.*, 2001; Naidu *et al.*, unpublished data) and this chapter reviews the results on the evaluation of vector resistance and investigates how this prevented transmission of the rosette virus agent complex. New recommendations in the process of identifying, evaluating and promoting virus and vector resistant varieties in key areas of SSA are also put forward.

The analysis of biological performance parameters of *A. craccivora* on ICG12991 under laboratory and field conditions provided sufficient evidence to suggest a strong aphid-resistance mechanism which was stable over time and under high aphid pressure (Chapter 2-3, 7). To elucidate the mechanism of aphid resistance in ICG12991, it was important to determine at which stage of the host plant selection (HPS) process the plants were rejected (Figure 1.13, Chapter 1). Direct observations of aphid probing behaviour under glasshouse and laboratory conditions showed that surface components at the epidermal layer were not obviously involved in the resistance mechanism of ICG 12991 (Chapter 5). Aphids readily inserted their stylets into the leaf tissue and the results indicated that a resistance factor was encountered after stylet insertion into the plant tissue of ICG12991. This finding is in agreement with suggestions that internal plant factors are the main cues used by aphids to accept or reject a plant (Pollard, 1973; Tjallingii, 1978b; Harrewijn, 1990; Montllor, 1991). A more detailed study of aphid feeding behaviour using the electrical penetration graph technique (EPG, direct current-system) and conducting experiments on honeydew collection provided sufficient evidence that feeding on ICG12991 was strongly affected irrespective of plant age

(Chapter 5). For example, the time from the start of the experiment for *A. craccivora* to locate and salivate into the phloem sieve elements of ICG12991, represented by the E1 waveform, was significantly longer than that taken by aphids probing on the susceptible control JL24. Additionally, salivation into the phloem of ICG12991 did not generally switch to ingestion (E2 waveform) which was frequently observed within a 4h recording period on JL24. Over longer feeding periods, phloem feeding on ICG12991 occurred but generally no honeydew was excreted within a 48h access period on the plants. This suggests that uptake of phloem sap was significantly reduced on ICG 12991 compared to on JL 24. The interference with sieve element location and phloem ingestion could therefore explain the low survival, slow development time and low fecundity of aphids confined on ICG12991 and rejection of these plants in choice tests (Chapter 2-3). It also explained why so few aphids were found on ICG12991 under field conditions in Uganda and the fewer and smaller aphid colonies on ICG12991 in Malawi compared to those on other varieties including JL24 (Chapter 3). Aphids which fail to feed under arid conditions in Africa may be more prone to mortality and may leave the plant after a few exploratory probes in search for a more suitable host.

The analysis of aphid feeding behaviour on ICG12991 also made it possible to relate aspects of aphid feeding with the results obtained on acquisition and transmission of the three virus agents of rosette disease; *Groundnut rosette assistor virus* (GRAV; Fam. *Luteoviridae*), *Groundnut rosette umbravirus* (GRV), and satellite RNA (satRNA) (Chapter 4). Successful transmission of rosette disease by the aphid vector and, consequently, the survival of the disease agents in nature depend on the intricate relationship among GRAV, GRV and satRNA. GRAV can only be inoculated into groundnut when *A. craccivora* salivates into the phloem sieve elements (Naidu *et al.*, 1999a) and all virus agents can only be acquired from the phloem (Robinson *et al.*, 1999), which requires sustained periods of phloem ingestion (Dubern, 1980; Misari *et al.*, 1988; Naidu *et al.*, 1999a). As mentioned before, phloem salivation and ingestion on ICG12991 were strongly inhibited and therefore the chance to acquire and inoculate the virus agents would be greatly reduced. The correlation between aphid feeding and virus acquisition and transmission could now explain the low disease incidence in ICG12991 compared to other varieties, which was repeatedly reported from field trials

in sub-Saharan Africa and the research centres ICRISAT-Malawi and SAARI-Uganda (Merwe van der & Subrahmanyam, 1997, Chiyembekeza *et al.*, 1997; Naidu *et al.*, 1999b; Subrahmanyam *et al.*, 2000).

The combined results on aphid behaviour and performance on ICG12991 and the transmission experiments emphasised the potential of vector resistant varieties as a component in new rosette disease control strategies. On the 3rd April 2002, ICG12991 was released as Serenut 4T in the Teso farming system of Uganda followed by its release in Malawi and known by the farmers as "new Erudurudu". This was a significant event since the varietal performance trials were accomplished within the project cycle, i.e within three years. Some commentators believe that this should be reduced to one season (Tripp 2003), but this carries risks, especially if a disease resistant line escapes exposure to the disease or adverse climatic conditions over one season. Prior to the release of ICG12991 in key areas of Africa, only virus resistant varieties were evaluated and included in breeding programmes (Gibbons, 1977; Bocklee-Morvan, 1983). ICG12991 has the advantage over current virus-resistant varieties of being a short-duration variety which allows two cropping seasons per year in many ecosystems throughout Africa (Naidu *et al.*, 1999b). In contrast, virus resistant varieties such as the recently released ICG SM90704 (GRV + satRNA resistant), also known as Serenut2 or Igola2, require a long growing season to attain maturity, making them susceptible to drought by the end of the season. Also the virus resistance in ICG SM90704 was not as effective as the vector resistance in ICG12991 during the transmission experiments (Chapter 4). Under high pressure of viruliferous aphids in the laboratory a large proportion of plants of ICG12991 remained free of all three virus agents, while almost all the plants of ICG SM90704 were totally infected. The plants were only 7 DAP at the time of the transmission experiments and this has most likely contributed to the relatively high infection of GRAV on ICG12991. Aphid performance and particularly their survival was significantly higher on young plants than on older plants and aphids were allowed 72 hours to inoculate the virus (Chapter 4). However, the three virus agents depend on each other for the perpetuation and spread of the disease in the field (Figure 1.7, Chapter 1) and the low transmission of all virus agents on very young plants of ICG12991 is a major advantage in designing control strategies

for GRD. Since the viral agents are not seed-borne, the primary infection must be introduced into the crop by viruliferous aphids and this would be greatly reduced on ICG12991 as opposed to on ICG SM90704. Planting ICG12991 may be particularly advantageous because farmers usually cannot follow recommendations on early sowing and close plant spacing to minimise early infection of viruliferous aphids. When primary infection is reduced, secondary spread of the disease would also be seriously hampered not just because of the relatively low number of infected plants of ICG12991 but more so because aphid feeding and therefore virus acquisition and subsequent transmission is significantly reduced. Additionally infected plants grow older during the period required for virus replication and aphids do not prefer mature plants for feeding and thus virus acquisition (Farrell, 1976a,b).

Various varieties with field resistance to rosette disease were aphid resistant (Chapters 2-3). This was assumed by a low number of aphid-infected plants and plants harbouring only small aphid colonies. They all originated from Asia where groundnut rosette does not occur and which emphasised the importance to screen the world's germplasm collection for vector resistance to control groundnut rosette disease. It also emphasised that there is a need to include vector resistant varieties in future breeding programmes to broaden the genetic base of resistance in groundnut and to enhance its durability against different variants of groundnut rosette virus agents. Breeding programmes have continued to focus on virus resistance but combining resistance genes to virus and vector in an elite line of groundnut would contribute to the sustainability of groundnut in SSA.

The success of vector resistance in groundnut to control the virus it spreads should encourage other research groups involved in pest management to promote research on resistance to insect vectors in varieties of various crops to control semi-persistent and persistent viruses. Due to the transmission characteristics of these viruses, breeding programmes for host-plant resistance to the vector could be focused on interference with various stages of the insects' host plant selection behaviour such as settling, probing, salivation and ingestion from the phloem. As opposed to control semi-persistent and persistent viruses, host plant resistance to the virus is only recommended to control non-

persistent viruses because these viruses may be transmitted within a single probe (Powell, 1991; Powell *et al.*, 1992). Spread of most non-persistent viruses is mainly through primary infections, especially when many viruliferous aphids enter the field (reviewed in Perring *et al.*, 1999) and plant resistance to the vector may therefore increase rather than decrease virus infections. This was illustrated in the field for the transmission of the aphid-borne *Cowpea mosaic virus* in the cowpea crop (Atiri *et al.*, 1984). Irrespective the use of virus- versus vector resistance, it is very important to elucidate the mechanism of resistance in order to avoid the loss of resistance genes during the breeding programmes (P.J.A. van der Merwe pers. comm.).

To better understand the aphid resistance mechanism in ICG12991 biological performance experiments and the EPG technique were particularly relevant (Chapter 2, 3, 5). It was already unequivocally illustrated that a resistance factor was encountered after aphids had inserted their stylets into the plant tissues and which interfered with sieve element location and phloem ingestion. Experiments however indicated that feeding was only affected on the leaf tissues of ICG12991. On the flowers, honeydew excretion illustrated that phloem feeding was neither delayed nor interrupted and was similar to that on susceptible control varieties. Undisturbed feeding on the flowers, as opposed to the leaves of ICG12991, could therefore explain the abundance of aphid colonies on the flower tissues in Malawi. This specific resistance expression in the leaf tissue on ICG12991 was also observed in the aphid resistant variety EC36892 (Padgham *et al.*, 1990b; Willekens & Kimmins, 2001). Analysis of EPG experiments and honeydew collection data showed that phloem ingestion from the leaf tissue of EC36892 was interrupted at almost regular intervals but not when feeding on the flowers. However, aphid feeding experiments on diets containing extracts of leaf and flower tissue of JL24 or ICG12991 did not provide evidence to suggest a constitutive chemical associated with leaf tissue of ICG12991 that caused deterrence. On the contrary, aphid probing was significantly stimulated over an 18h period on all diets containing leaf and flower extracts of either a resistant or a susceptible variety, which was reflected in a significant increase in total number of probes and length of probes recorded in the diets (Chapter 6). Aphid feeding experiments also illustrated that *A. craccivora* could detect chemical compounds within a gel of agarose (1.2 % w/v) which

was designed to simulate the mesophyl area of plant tissues (Chapter 6; Urbanska *et al.*, 1998). Therefore it was suggested that aphids can detect internal plant stimuli during pathway activity before reaching the sieve elements. Apoplastic factors, either constitutive or induced during the stylet pathway activity could guide the aphid stylets towards the sieve elements as opposed to a trial-and-error procedure in which every cell encountered along the intercellular pathway is punctured (Tjallingii & Hogen Esch, 1993). A physical barrier which inhibited stylet pathway progression or which prevented the aphids to initiate salivation into the phloem were also not likely because within a probe that led to phloem salivation on ICG12991, the time to do so did not differ than on the susceptible JL24.

To summarise the results of the attempts to elucidate the resistance mechanism in ICG12991 we could state that:

1. plant surface components were not associated with the aphid resistance mechanism;
2. resistance was encountered by the aphids after stylet insertion into the plant tissue and prior to phloem sieve element location;
3. no constitutive probing or feeding deterrent nor a physical barrier could be related to resistance;
4. resistance was expressed in leaf, but not the flower tissues of ICG12991.

It was concluded that a plant defence mechanism in ICG12991 was induced on leaf tissue by probing aphids and that the resistance factor was perceived during stylet pathway activity which then influenced aphid feeding behaviour.

The conclusion that the resistance in ICG12991 was induced rather than constitutive was further supported because around the aphid feeding sites localised areas of cell collapse were observed. These areas were also identified on 6 other Asian varieties which expressed resistance to *A. craccivora* (Chapter 2) and which were reported to be field resistant against groundnut rosette (Subrahmanyam *et al.*, 1998). The rapidity of cell collapse and/or cell death, the early and local accumulation of phenolic compounds

around the feeding sites (<24h) and the strong association of this induced response with aphid resistance suggested that it can be interpreted as a true hypersensitive response (Alston & Briggs, 1970; Lyth, 1985; Massonié *et al.*, 1981; Miles, 1999). Two distinct types of hypersensitivity were observed. On ICG12991 small areas of cell collapse were visible as "white lesions" on the adaxial leaf surface, after the aphids had probed on the abaxial leaf surface. This plant response was also identified on the aphid resistant variety ICG SM99540. The second type was associated with distinct areas of necrosis around the aphid feeding sites and observed on five aphid resistant varieties including EC36892, ICG9723, ICG11735, ICG11788 and ICG11649. Necrosis is more common as a resistance expression to phloem feeding insects either surrounding the penetration site or by systemic action at some distance from it (Miles, 1999). ICG12991 and ICG9723 were selected to characterize the hypersensitive response (HR) and to compare the two types of expression. EC36892 would have been the preferred variety to select because data were already published and available on the behaviour of *A. craccivora* on this variety (Padgham *et al.*, 1990a,b). However, bad seed germination rates and growing characteristics prevented further work with this variety. Preliminary analysis of aphid behaviour on ICG9723 showed comparable results with those published in the literature on EC36892. The complex behaviour of *A. craccivora* and the similarities and differences of aphid feeding on ICG12991 and ICG9723 as opposed to on a susceptible variety are summarized and the characteristics of both types of HR are presented (Table 8.1).

Table 8 1: Similarities and differences between the hypersensitive response to aphid feeding on ICG12991 and EC36892/ICG9723 when compared to susceptible varieties.

Parameters for comparison	**ICG12991**		**EC36892/ICG9723**	
Aphid resistance	Strong		Moderate	
Aphid development time	Increased		Increased	
Aphid fecundity	Reduced		Reduced	
Aphid survival	Reduced		Not reduced	
Plant rejection in choice test	1-3 hours		10-12 hours[1]	
	Leaf	**Flower**	**Leaf**	**Flower**
Time to 1st salivation into phloem sieve elements	Delayed	Not delayed	Not delayed[1]	Not delayed[2]
Ingestion form phloem within 4h recording period	Not observed	*	Interrupted[2]	Continuous[2]
Time to 1st honeydew collection from start of experiments	Delayed	Not delayed	Not delayed	Not delayed
Honeydew collection	Minimal	Continuous	Interrupted	Continuous
Hypersensitivity	Expressed	Not Expressed	Expressed	Not Expressed
Necrosis	Microscopic	Not expressed	Macroscopic	Not Expressed

[1] Padgham *et al.*, 1990b.

[2] F.M. Kimmins unpublished data

It was already discussed that the delay in phloem accession and inhibition of sustained feeding was correlated with poor aphid performance and survival on ICG12991. In contrast, aphids showed periods of sustained ingestion on EC36892 and ICG9723 which could be correlated with higher aphid survival and performance on these varieties. Research to further investigate the mechanism on EC36892 were ceased in the early 1990s because the variety did not have the growing characteristics nor marketable traits desired by farmers in Africa. The accumulated data on aphid performance, aphid feeding behaviour through direct observation, EPG and honeydew collection and transmission studies of the groundnut rosette virus agents undoubtedly correlated hypersensitivity with aphid resistance and indirect virus resistance. It remains to be determined whether aphids feed long enough on ICG12991 in the field to induce hypersensitivity because plants were quickly rejected. However it was observed that the response can be expressed within a onehour aphid feeding period in the laboratory and salivation into the phloem sieve elements was not a requisite (Chapter 5). It was

proposed that early events leading to plant hypersensitivity and cell collapse or cell death are induced and eventually perceived by feeding aphids during pathway activity in the mesophyll area.

The hypersensitive response has been considered as an important type of an induced plant defence mechanism whereby the plant elicits a response to fungi, bacteria, virus, nematode, mite or insect attack (Paulson & Webster, 1972; Maclean *et al.,* 1974; Agrios, 1988; Fernandes, 1990; Grover, 1995; Gopalan *et al.*, 1996; Low & Merida, 1996; Fritig *et al.*, 1998). The mechanisms involved to generate the hypersensitivity and ultimately causing resistance have been subjected to intensive research and the most complete picture we have is the HR in response to plant pathogenic bacteria (reviewed in Jabs & Slusarensko, 2000). The HR is generally accepted under the definition of "*rapid death of plant cells associated with disease resistance*" (Stakman, 1915; Goodman & Novacky, 1994; Greenberg, 1996; Heath, 1998) and encompasses all morphological and histological changes that are expressed (Müller, 1959). Hypersensitivity induced by plant pathogens and insects is generally associated with membrane damage and electrolyte leakage, oxidative burst (production of Reactive Oxygen Species (ROS)) and finally cell collapse and death.

Our understanding of hypersensitivity and associated oxidative stress as a mediating factor in insect-plant interactions is still in its infancy (Ahmed, 1992) because studies of plant resistance to insects had been centred on a wide spectrum of plant features such as secondary compounds, nutritional factors, phenology, age, induced defence, morphological traits, tissue hardness, colour, shape and size etc... (reviewed in Fernandes, 1990). Progress in oxidative stress in entomological research had been hampered by the misconception that oxidative stress is a slow-acting process and may be relatively insignificant for animals possessing short life-spans such as aphids. However, oxidative activities are a natural part of the plant's defence and a redox system controls the oxidation rates during the responses of plants under attack by sucking insects such as aphids (Miles & Oertli, 1993). The first oxidation products of phenolic compounds are generally deterrent to insects and in several cases now, plant resistance to herbivores has been correlated with an enhanced oxidative state of plant

tissues (Felton *et al.*, 1992a,b; Chiang *et al.*, 1987; Nuepane & Norris, 1991a,b; Kanofsky & Axelrod, 1986; Chamulitrat *et al.*, 1991; Shukle & Murdock, 1983; Mohri *et al.*, 1990; Bi *et al.*, 1994). Hypersensitivity, induced by aphids is reported in various systems such as apple (Alston & Briggs, 1970; Lyth, 1985), peach (Massonié *et al.*, 1982) and groundnut and could indicate a common defence system in plants to phloem feeding insects. Its potential as a management strategy against insect-vectored diseases has been overlooked. Aphids that were reared for over a hundred generations on the resistant ICG12991 still induced the hypersensitive response and they did not transmit the virus agent more efficiently than a control population. Low transmission of GRAV could still be correlated with phloem sieve element location as described earlier, but the process of cell collapse associated with the hypersensitive response could have been responsible for the low infection of GRV and satRNA on ICG12991. GRV and satRNA can be inoculated during exploratory probes in the mesophyl which undergo hypersensitivity and hypersensitivity has been more than once reported to limit virus replication and spread in plants. More specifically; "*the HR involves the extremely rapid death of only a few host cells, which limits the progression of the infection and arrests the disease*" (Goodman & Novacky, 1994). In contrast, the resistance mechanism could lead to an increased probing frequency and therefore increase inoculation rates of viruses. An increased branching pattern of stylet paths was already reported for aphids feeding on EC36892 (Padgham *et al.*, 1990b) but these authors did not mention the hypersensitive reaction. The poor growth characteristics of this variety masked this reaction (F.M.Kimmins pers comm.). The branching pattern of the stylet sheaths in EC36892 show great similarity with increase branching of fungal hyphae into intercellular spaces around dead plant cells associated with hypersensitivity (Stakman, 1915; Richael & Gilchrist, 1999). Nonetheless, field data have consistently shown a low virus infection of all three agents of rosette disease on HR expressing varieties EC36892 and more importantly on ICG12991 (Padgham *et al.*, 1990b; Subrahmanyam *et al.*, 1998; Chiyembekeza *et al.*, 1997).

A more detailed comparison between induced plant responses to insect herbivory and pathogens may shed some light on how aphid feeding and induction of hypersensitivity can be correlated or how aphid feeding can induce a plant defence mechanism originally

ascribed to plant pathogens (Appendix 5). Recently signal interactions in induced plant responses to pathogens and insect were compared and, as already indicated, HR-induction by aphids is not well developed yet (Fidantsef *et al.*, 1999). Examples from the literature are cited to emphasise the overlap in signal transduction pathways induced by insects and pathogens leading to hypersensitive related plant reaction and the need to continue research on the characterisation of the HR in ICG12991 induced by *A. craccivora*.

Induced resistance to insect attack is usually mediated by the jasmonate-wound-signal transduction cascade characterised by proteinase-inhibitors, PI (Stout *et al.*, 1994), whereas pathogen-infection, whether or not resulting in a hypersensitive response, is mediated via the salicylic acid pathway characterised by pathogen-related proteins, PR (Hammerschmidt, 1999; Sticher *et al.*, 1997; Durner *et al.*, 1997; Loon van *et al.*, 1998). Both pathways are activated within minutes of elicitation (Schittko *et al.*, 2000; Chen *et al.*, 1995) and recent research does not support the hypothesis of a strict dichotomy of signalling by insects and pathogens (Fidantsef *et al.*, 1999). Overlaps in the response pathways have been illustrated by aphids feeding on tomato leaves *Lycopersicon exculentum*. The aphids *Myzus persicae*, Sulzer and *Macrosiphum euphorbiae*, Thomas induced PR proteins in a similar way to a fungal infection and fungal elicitor, arachidonate-acid whereas chewing insects or jasmonic acid did not (Cohen *et al.*, 1991). Also mites (*Eriophyes cladophthirus,* Nal.) induced PR proteins on *Solanum ducamara* L. which accumulated in the symplast and apoplast of infested leaves expressing hypersensitivity (Bronner *et al.*, 1991). PR-proteins were already accumulated within 12h of mite infection whereas lesions were already detectable within 1h of mite attack. Lesions on groundnut variety ICG12991 and hypothesised accumulated phenolics were detected within 24h of aphid attack but it was suggested already that *A. craccivora* probably detected early signals of the HR while probing the mesophyll. Early signals could include phenolic oxidation products and reactive oxygen species or lipoxygenases which participate in the peroxidation of membrane lipids and synthesis of signalling molecules (Hamberg & Gardner, 1992; Siedow, 1991). In addition, peroxidases and polyphenol oxidases are known aphid salivary enzymes which are thought to be injected to change the natural redox equilibrium in uninfested

plants and induce the oxidation of phenolics which are maintained in a reduced state by anti-oxidants (Miles & Oertli, 1993). The first oxidation products may be toxic to the insect and further oxidation result in non-toxic insoluble phenolic polymers (Peng & Miles, 1988) and could resemble the accumulation of phenolics as detected in varieties ICG12991, EC36892 and ICG9723, all expressing hypersensitivity to aphid feeding.

There is sufficient evidence in the literature to support the hypothesis that aphids and other plant sucking insects can induce a plant defence mechanism in a similar way as ascribed to plant pathogens, but exactly how it is elicited is not clear and it may vary from species to species and from host to host. The example of hypersensitivity in groundnuts is one more in an increasing list of case studies but could become a model to characterise the response. Pohlon & Baldwin (2001) provided a way to 'freeze' a plant's chemical dynamics in artificial diets and to study feeding behaviour in response to induced substances. The successful design of feeding bioassays for *A. craccivora* would allow not only a study of its salivary enzymes, which may induce the response, but also a study of aphid feeding in relation to the induced hypersensitivity in ICG12991.

There is a related study in progress on the marker-assisted selection of vector resistance in ICG12991 at the ARC, Grain Crop Institute, South-Africa (L. Herselmann pers. comm.), but to date few polymorphisms have been detected between different varieties with substantial variation for phenotypic traits such as plant growing habit, seed colour and size, and resistance to biotic and abiotic factors (He & Prakash, 1997). In the absence of these tools to rapidly select for vector resistance in future breeding, physiological markers related to hypersensitivity could be used. In the future however, molecular markers may be found in cultivated groundnut (*Arachis hypogaea*, L.) to detect variation at the DNA-level.

In the absence of molecular markers to detect valuable traits in groundnut the screening method developed at ICRISAT-Malawi to evaluate the world's groundnut germplasm for resistance to groundnut rosette disease (GRD) is still the only mechanism which can identify high yielding genotypes with good levels of rosette resistance (Bock, 1987;

Figure 1.1). Although this screening method identified rosette resistant varieties for over 15 years, shortcomings apart from labour-intensity, have become apparent now the aetiology of the disease is better understood (Chapter 1 and references therein). The assessment of rosette disease incidence at the end of the growing season in screening trials is solely based on the expression of disease symptoms, which is indicative for GRV and satRNA but does not allow identification of GRAV-infection and resistance (Murant *et al.*, 1988). This means that to date, no groundnut accession expressing resistance to GRAV has been identified or rather recognised because on its own, GRAV does not cause symptoms. Recently, GRAV resistance was reported in wild species of groundnut and it was argued that methods to introgress resistance genes from wild species into cultivated species needed to be exploited (Subrahmanyam *et al.*, 2001). However, the report has not investigated whether the absence of GRAV in accessions was due to plant resistance to GRAV or through resistance to the aphid vector. Therefore failure to detect GRAV in plants by diagnostic tests such as RT-PCR and TAS-ELISA does not necessarily mean resistance or immunity to this agent. The lines could have been screened using the diagnostic tests but these are expensive to use in a mass trial. Promising lines could be selected and a combination of grafting and use of RT-PCR or TAS-ELISA could be used to identify GRAV resistant material. Unfortunately there are no plans to do this and current donor activities (e.g. USAID) are focusing on the development of transgenic resistance, specifically GRAV-coat protein mediated resistance.

The infector row technique nonetheless remains the most practical way to assess GRD incidence. However, screening GRD incidence by symptoms only has almost always overlooked plant resistance to the vector as an indirect resistance to the disease (Chapter 4). Observations on vector populations at the screening trials should therefore become an integral part of the screening process. Aphid colonies and their size were reliable indicators for aphid resistance in groundnut and the assessment was also a relatively quick method (Chapter 3). Additionally, small-scale experiments in the glasshouses would rapidly complement the field observations. Glasshouse experiments would be more often required at the stations where screening occurs under natural conditions, such as SAARI-Uganda because test varieties may escape aphid infestation and low

disease incidences may not always be reliable, especially in seasons of low rosette incidence.

In conclusion, the research on rosette is benefiting smallholder farmers as they are able to grow a valuable crop with reduced risk to pests and diseases. Farmers are now multiplying the improved varieties and benefiting from the increased household incomes.

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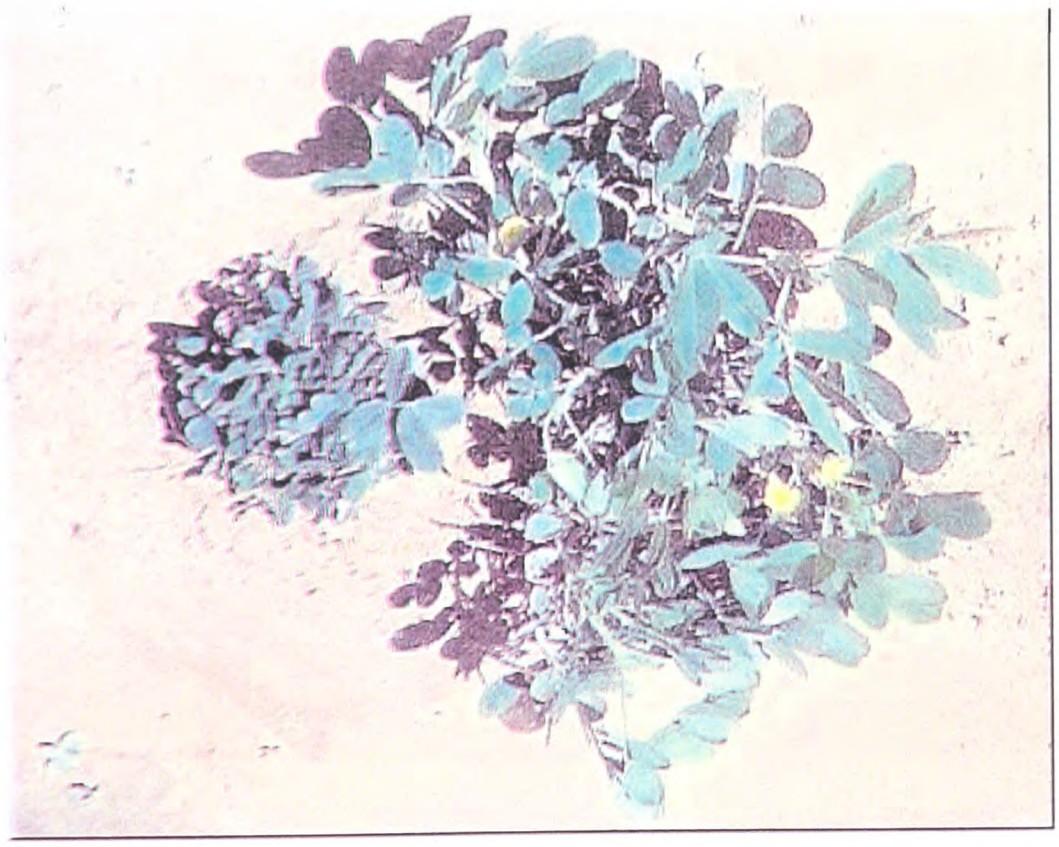
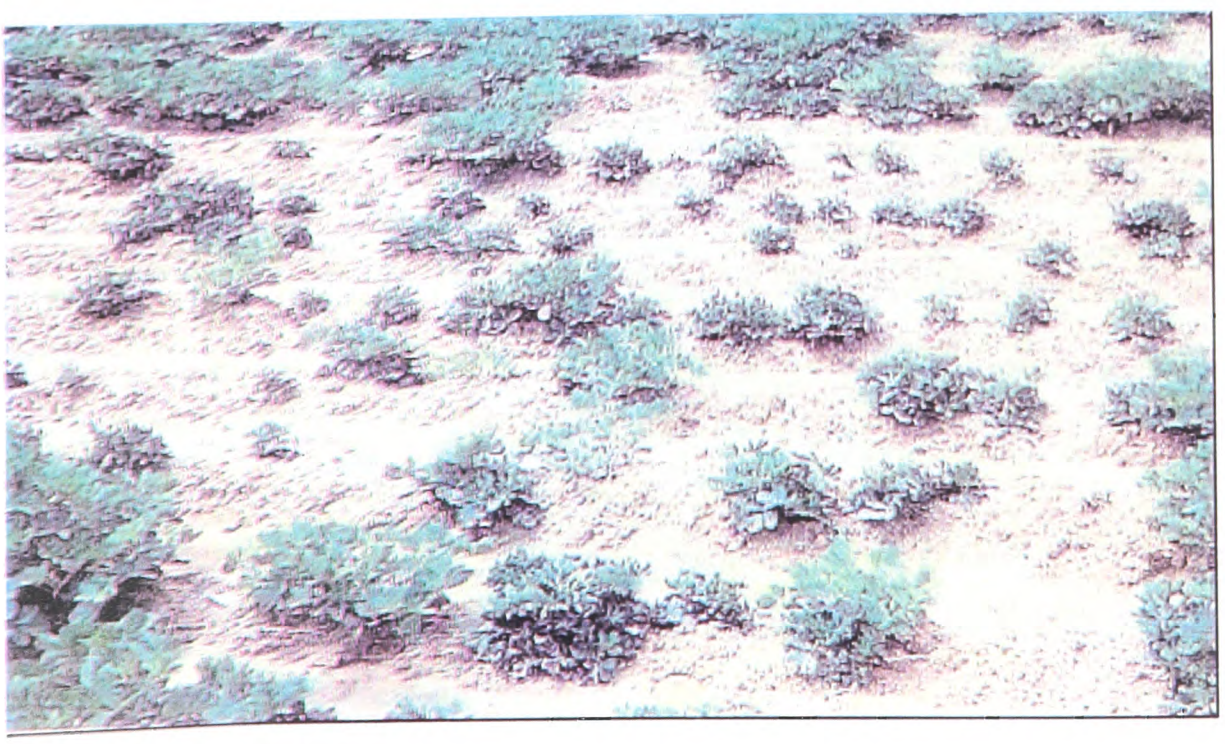
APPENDICES

Appendix 1a: Groundnut plant showing symptoms of the green variant of groundnut rosette virus disease. The plant on the left is the virus infected plant while the plant on the right is a healthy one.



Appendix 1b: The green variant of groundnut rosette virus disease in the field.



Appendix 2a: Groundnut varieties in field experiments at SAARI-research station in Uganda were labelled A to J and until final analysis it was unknown which variety was linked with which letter. Letter F corresponded to groundnut variety ICG12991

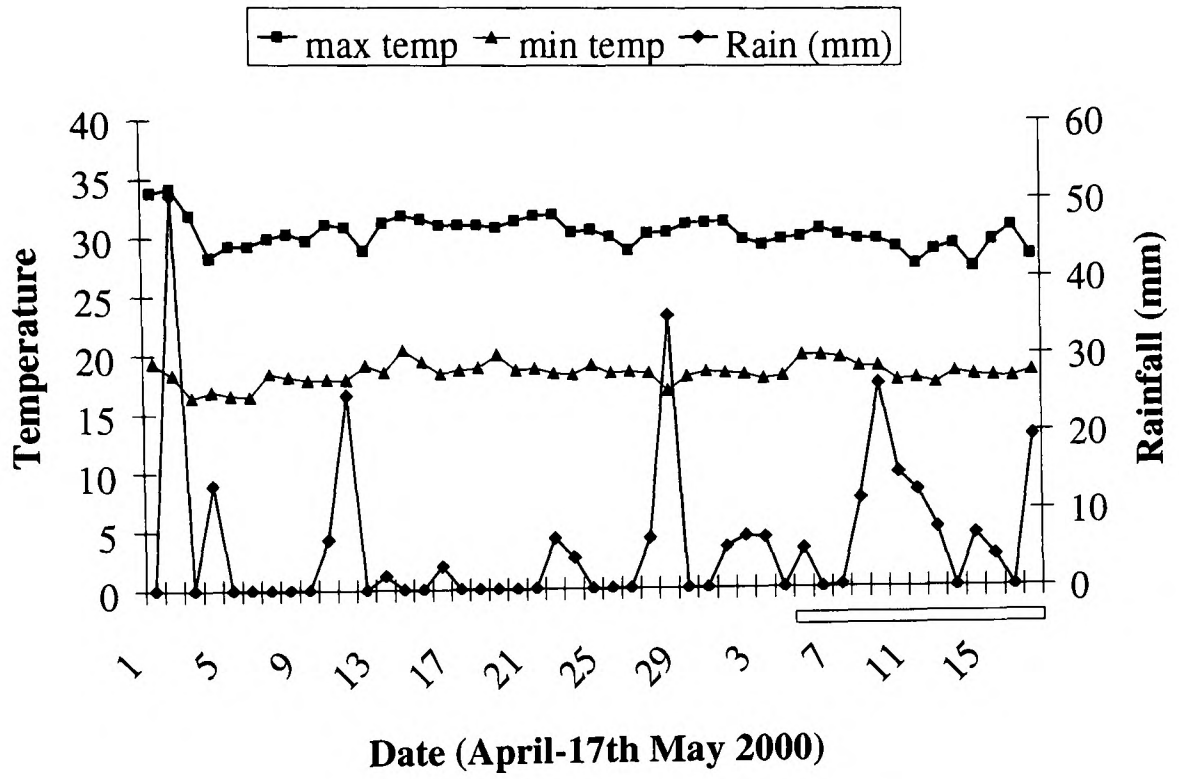
Appendix 2 b: Ten varieties were planted in a randomised block design, 6 rows of plants per variety, 5m long and uniform spacing of 45cm.

Aphid counting in progress by Mr. Bill Page

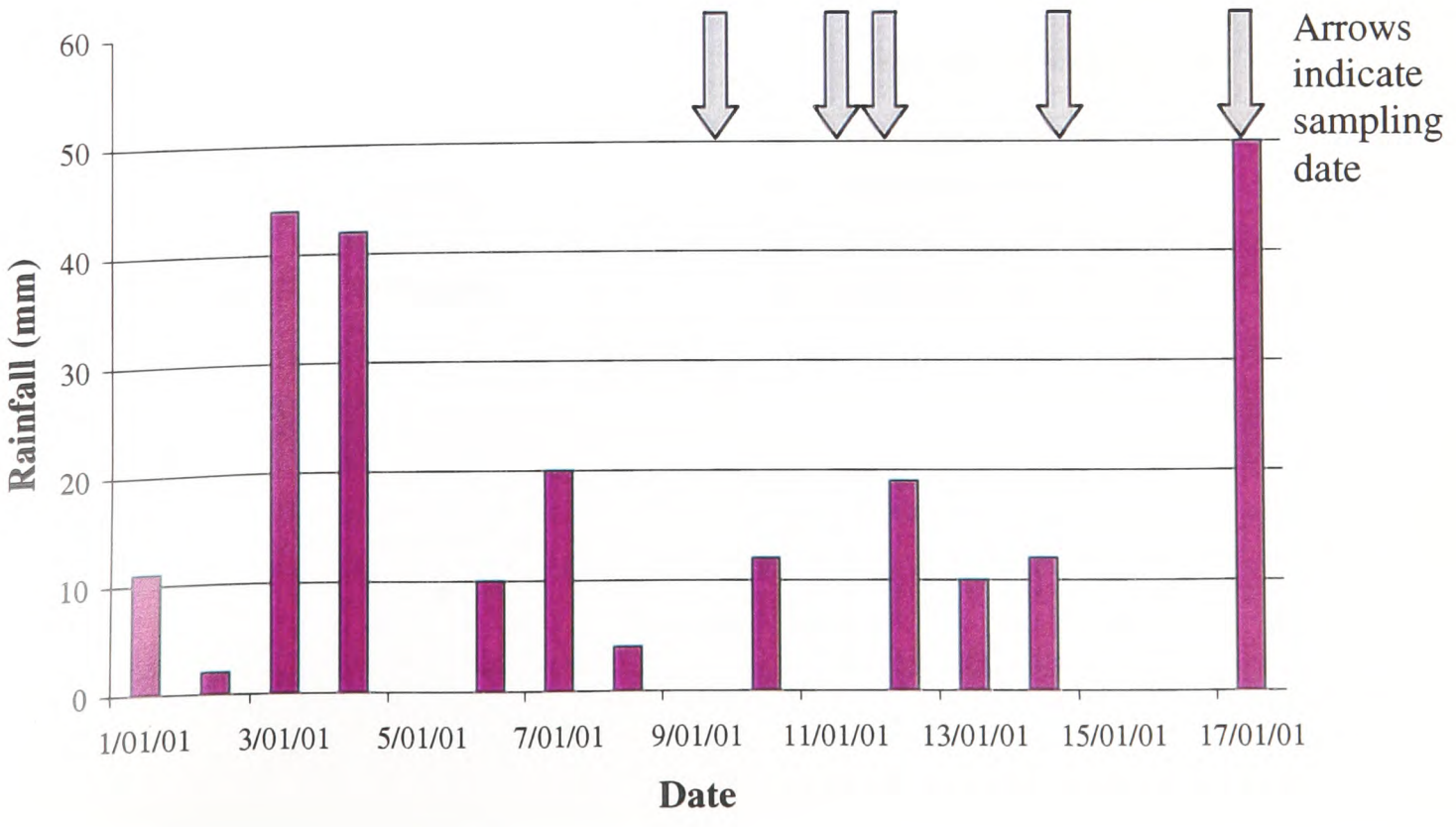
Sticks to indicate the various plots of varieties

Appendix 3: Groundnut plants were enclosed in two crisp bags to contain the aphids during the experiment, to prevent natural infestation and to protect the set-up from splashing mud during periods of rain. The plants were additionally protected from heavy rain with polystyrene tiles.

Appendix 4a: Temperature and rainfall recordings from April 1st until May 17th 2000 at the Serere Agricultural and Animal production Research Institute, Uganda. Plants were planted on April 17th and the grey rectangle denotes when the experimental work was conducted.



Appendix 4b: Rainfall pattern from January 1st until January 17th as obtained from the rain-gauges placed into the experimental plots at the International Crops Research Institute, Malawi.



Appendix 5: Model for generation of signals to engage different local systemic stress responses following pathogen and insect attack. ROS: Reactive Oxygen Species; HR: Hypersensitive Response; (adapted from Fidantsef *et al.*, 1999).