UNIVERSITY OF HANOVER

DEPARTMENT OF HORTICULTURE

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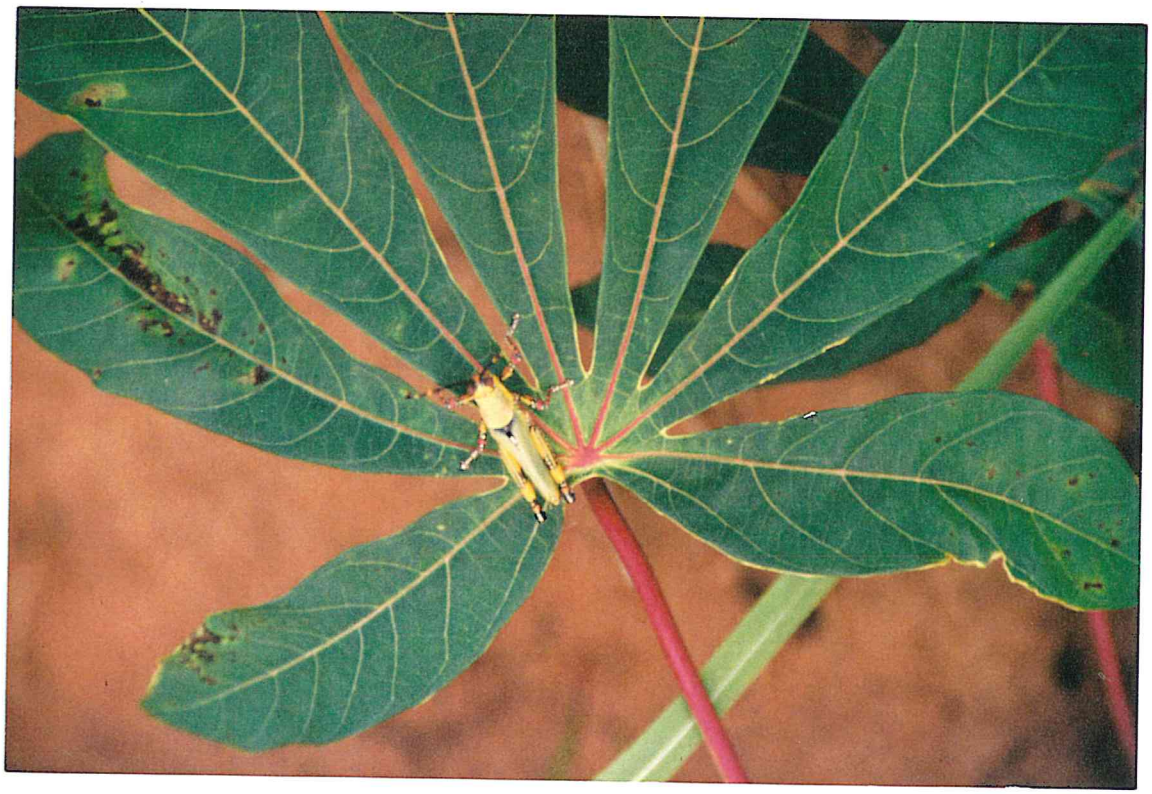
THE ROLE OF INSECTS AS POTENTIAL VECTORS OF CASSAVA AND COWPEA BACTERIAL BLIGHT IN WEST AFRICA



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Dedicated to my late parents, my husband and Katché, my son

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Abbreviations

CBB	cassava bacterial blight
CoBB	cowpea bacterial blight
CoBP	cowpea bacterial pustule
Cfu	colony forming unit
g	gram/gravity
h	hour
mg	milligram
$MgSO_4$	magnesium sulphate
µ	micrometer
M	mole per litre
min	minute
ml	millilitre
l	liter
NGA	nutrient glucose agar
Rpm	rotations per minute
SSM	Semi-selective medium
t/ha	tonne per hectare
pm	post meridian
GSPB	Göttinger Sammlung Phytopathogener Bakterien

Abstract

Xanthomonas campestris pv. *manihotis* (Bondar, 1912) (*Xcm*) and *Xanthomonas campestris* pv. *vignicola* (Burkholder, 1944) (Dye, 1978) (*Xcv*) are both worldwide important pathogens, causing bacterial blight diseases of cassava and cowpea, respectively. The presence of the pathogens in/on the variegated grasshopper *Zonocerus variegatus* (L.) (Orth.: Acrididae), an occasionally important pest of cassava and cowpea in West Africa, was demonstrated. *X. campestris* pv. *manihotis* and *X. campestris* pv. *vignicola* were detected on the legs, the mandibles, in the intestines and in the faeces of the insects by isolation. Additionally *X. campestris* pv. *vignicola* were isolated from two grasshoppers species (*Pyrgomorpha cognata* (Krauss), *Oedaleus spp.* (Fieber)), one Hymenoptera (*Apis mellifera* (L).), and three Coleoptera (*Ootheca mutabilis* (Stahl), *Milabris spp.* (Fab.) and *Exochomus troberti* (Mulsant)). After one week of acquisition period, the indirect immunofluorescence showed that *X. campestris* pv. *manihotis* was present in the insect gut long after the initial acquisition period. Infection resulted when *Z. variegatus* specimens that had previously fed on cassava bacterial blight-infected plant material were transferred to healthy cassava plants. Also, damaged and non-damaged cassava plants showed symptoms when they were treated with *X. campestris* pv. *manihotis*-contaminated *Z. variegatus* faeces. However, attempts to transmit *X. campestris* pv. *vignicola* via *Z. variegatus* specimens that had previously fed on cowpea bacterial blight-infected plant material failed. Yet, the survival of bacteria as epiphytic population and the contamination of other insects suggest the possibility of insects vectoring cowpea bacterial blight.

1 Introduction and literature review

Cassava (*Manihot esculenta* Crantz, Euphorbiaceae) is a major staple for more than 400 million people in Africa, South America and Asia (Cock, 1982; El-Sharkawy, 1993). Sub-Saharan Africa is with 82 million tonnes per year the biggest cassava producer in the world (FAO, 1996). Average yields in Africa are around 12 t/ha, with a range of less than 1 to more than 67 t/ha (Nweke et al., 1994).

Cassava is rather tolerant to drought and low soil fertility. It is primarily grown for subsistence by small-scale farmers in areas with poor soils or unfavourable climate. The protein-rich leaves are widely consumed as vegetables in Central Africa, and also in Benin, though cassava is primarily grown for its starchy roots. The roots produce more calories per unit of land than any other crop in the world, except sugar-cane (Nweke, 1996). Besides its role in food security, in Africa cassava serves as a source of cash income for small farmers and as main carbohydrate source for the low-income urban population (Lynam, 1991; El-Sharkawy, 1993; Nweke, 1994). On a worldwide basis, approximately 2/3 of the production is intended for human consumption, while the remaining 1/3 is used as animal fodder and for industrial purposes (Nweke, 1996).

However, cassava suffers from numerous bacterial and fungal diseases some of which are important constraints to the production and marketability of the crop. Cassava bacterial blight (CBB) caused by *Xanthomonas campestris* pv. *manihotis* (*Xcm*) is the second most important disease after cassava mosaic virus disease in Africa. The pathogen recently renamed *Xanthomonas axonopodis* pv. *manihotis* (Vauterin et al., 1995), was first reported in Brazil (Bondar, 1912). Since the 1970, it has been observed in various African countries (Terry and Ezumah, 1974; Lozano and Sequeira, 1974). Recent surveys revealed the prevalence of the disease in Benin and Nigeria, with regionally severe outbreaks (Wydra and Msikita, 1998).

Significant yield losses were observed in several ecozones of West Africa, occasionally exceeding 50% (Wydra and Rudolph, 1999; Wydra et al., 2001).

The pathogen invades its host systemically, and the transmission by vegetative propagation is often overlooked. It causes various symptoms, such as angular leaf spots, leaf blight (Fig. 1.1), leaf wilt, tip dieback and lesions on stems with production of exudates (Fig. 1.2) (Lozano and Sequeira, 1974; Lozano, 1975; Daniel and Boher, 1981a, 1981b).

Cassava plants are attacked by many insects such as cassava mealybug (*Phenacoccus manihotis* (Mat. Ferr.) (Hom.: Pseudococcidae)), cassava green mites (*Mononychellus tanajoa* (Bondar) (Acari.: Tetranychidae)), cassava scales (*Aonidomytillus albus* (Cockerell) (Hom.: Diaspididae)) and the variegated grasshopper *Zonocerus variegatus* (L.) (Orth.: Acrididae).

Zonocerus variegatus, is a Pyrgomorphidae widely distributed in the West and Central African humid forest and savannah zones. It is a polyphagous species for which a considerable bibliographic database exists (Boppre et al., 1984; Chapman et al., 1986; De Gregorio, 1989; Chiffaud and Mestre, 1990). The infestation of cassava by *Z. variegatus* has been reported (Bernays et al., 1975; McCaffery et al., 1978; Launois-Luong, 1979; Bani, 1990b). Among the Acridoidae, *Z. variegatus* is the most important pest species. In general, acridoids feed on leaves and the green stem. Adults and nymphs defoliate the plant and sometimes strip the bark, if infestation is high. Preliminary field observations indicated that the *Z. variegatus* prefers cassava bacteria blight-infected leaves (Bani, 1990b).

Besides cassava, cowpea (*Vigna unguiculata* (L.) Walp, Fabaceae) is another major food crop cultivated in tropical and sub-tropical regions of Africa, Asia, Central and South America and Southern Europe. Worldwide cowpea is produced on approximately 6 million ha, with an average dry grain yield of 240 kg/ha, and average green pod yield of about 28-29 t/ha (Quin, 1997; Allen et al., 1998). The largest cowpea producers in the world are Nigeria,

Brazil, India, and several other African countries (Singh and Rachie, 1985). The crop is grown for its young pods, green or dry seeds and young leaves. The protein content in the mature seeds is about 25% and the starch content 60%. Thus, cowpea is a cheap protein source for the poor population of the world. Cowpea is a good forage and cover crop with a nitrogen fixation capacity of up to 240 kg/ha, while up to 60 to 70 kg N/ha remains for the succeeding crop. The crop is highly compatible as a companion crop and in Africa is usually intercropped with maize, cassava, millet, groundnut and sorghum.

The most important bacterial disease of cowpea is cowpea bacterial blight (CoBB) caused by *Xanthomonas campestris* pv. *vignicola (Xcv)* (Burkholder, 1944; Dye, 1978). First described in Oklahoma, USA, in 1931 (Brillhart, 1934), the disease is prevalent in all major cowpea-growing areas in the world (Emechebe and Florini, 1997). Grain yield loss can be higher than 64% (Wydra and Rudolph, 1999). The plant may be infected at different growth stages and highly susceptible cultivars may completely be destroyed (Sheerwin and Lefebvre, 1951; Emechebe and Shoyinka, 1985). Symptoms of cowpea bacteria blight infection appear as tiny, water-soaked, translucent spots, which enlarge, coalesce and develop to large necrotic areas, usually with a yellow halo, leading to premature leaf drop (Fig. 1.3). The disease systemically invades stems and seeds. The pathogen may also cause pustule-like symptoms (Khatri-Chhetri et al., 1999) on the abaxial surface of leaves, visible as tiny, dark, raised, translucent and water-soaked lesions, which may enlarge to about 3 mm diameter.

A great variety of insects attack leaves, stems, pods and flowers of cowpea. Severe damage appears during the flowering stage and podding (post-flowering). Pests include flower thrips, predominantly *Megalurothrips sjostedti* Tryb (Thysa.: Thripidae), the legume pod borer (*Maruca vitrata* Fab. (Lep.: Pyralidae), and a complex of pod and seed suckers, with *Clavigralla tomentosicollis* Stål (Hem.: Coreidae) as the most important species (Singh et al.,

1990). *Empoasca* spp. (Hom.: Cicadelidae), *Ootheca mutabilis* Sahlberg (Col.: Chrysomelidae), *Anoplocnemis curvipes* (Fab.) and *Riptortus dentipes* (Fab.) (both Hem.: Coreidae) are also mentioned as occasional pests of cowpea (Singh et al., 1990). During preliminary surveys, *Z. variegatus* was frequently found in cowpea fields, causing serious damage to the leaves (M. Zandjanakou, unpublished results).

Many insects have been observed to carry plant pathogenic bacteria (Kloepper et al., 1981; Barbehenn and Purcell, 1993; Wayadande and Fletcher, 1995; Myoung-Ok et al., 1999; Damon, 2000). Purcell, (1982) reported that vector-pathogen relationships are important components of the epidemiology of many plant diseases. In the recent literature, little vector specificity was found for plant pathogenic bacteria, and, thus, combating vectors is often regarded as a less promising control strategy against bacterial diseases. For instance an enormous diversity of possible insect vectors were observed for *E. amylovora* (Burr.) (Winslow et al.) (Harrison et al., 1980; Van der Zwet and Keil, 1979). For Pierce`s disease or clover club leaf and plant diseases caused by mollicutes such as *Spiroplasma citri* J. Gen, insect vectors are generally necessary as intermediate hosts (Purcell, 1982). They may contribute to the survival of the pathogens, and disseminate both primary and secondary inoculum of the pathogen from plant to plant (Walter and Vakili, 1977). Assistance in survival, dissemination and penetration of host tissue are the most important ways in which insects interact with plant pathogenic bacteria. When this assistance is combined with the natural advantages of bacteria over other pathogens because of their high reproduction capacity, rapid entry into infection courts and independent motility, bacterial pathogens can become extremely destructive (Harrison et al., 1980).

Zonocerus variegatus has been reported to successfully transmit cowpea mosaic disease (Whitney and Gilmer, 1974), okra mosaic disease (Givord and Den Boher, 1980) and cassava bacterial blight (Bani, 1990b).

For the development of sustainable control measures against *X. campestris pv. manihotis* and *X. campestris pv. vignicola* detailed knowledge on the epidemiology, the inoculum source and ways of dissemination of the pathogens is a prerequisite.

Our hypothesis is that insects in general, and *Z. variegatus* in particular, are vectors of cassava and cowpea bacterial blight. In this study we tried to elucidate the role of insects in dissemination and transmission of bacterial blight of cassava and cowpea. The specific objectives were:

1 to study the survival of *Xanthomonas campestris pv. manihotis* in/on insects,

2 to elucidate the transmission of *Xanthomonas campestris pv. manihotis* and *Xanthomonas campestris pv. vignicola* by insects,

3 to determine the inoculum level necessary for symptom development,

4 to localize *Xanthomonas campestris pv. manihotis* in insects by means of immunofluorescence microscopy and

5 to determine the influence of *X. campestris pv. manihotis* on the development of *Z. variegatus*.

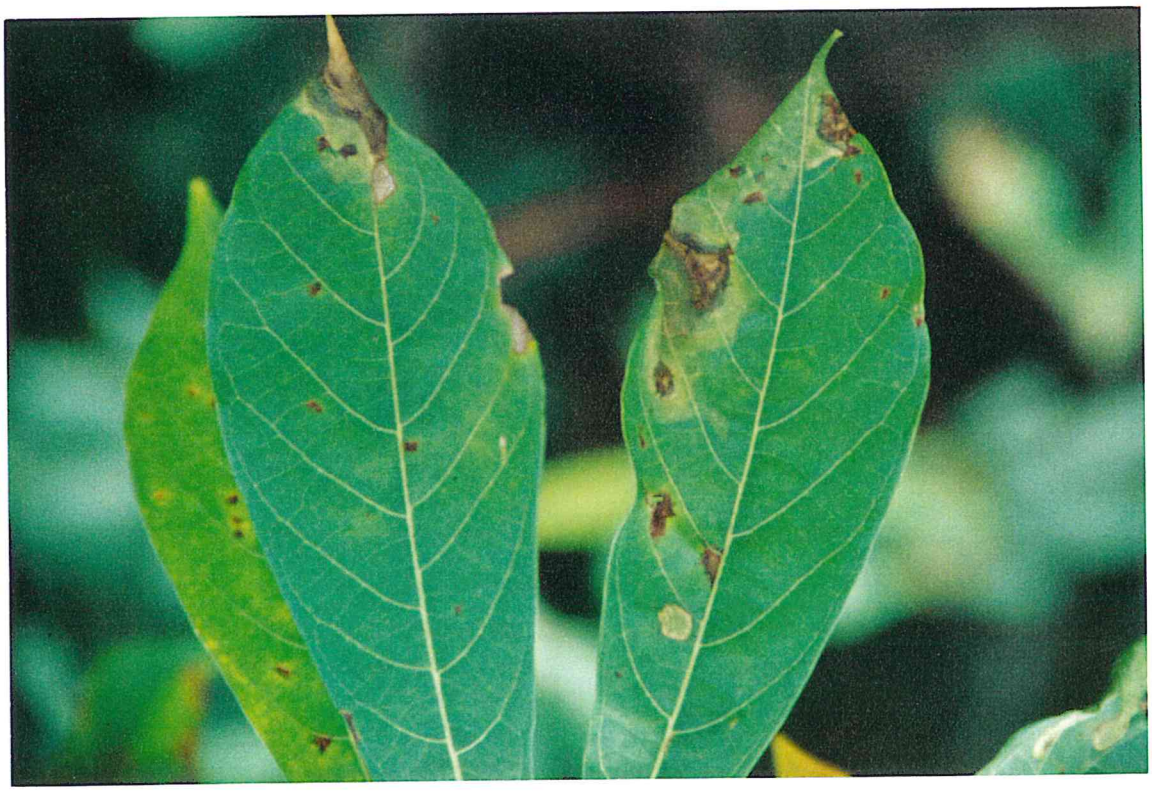

Fig. 1.1: Symptom of water-soaked leaf spots and blight on cassava caused by *X. campestris* pv. *manihotis*

Fig. 1.2: Exudate symptoms on cassava caused by *X. campestris* pv. *manihotis*

Fig. 1.3: Symptoms of bacterial blight and wilt on cowpea caused by *X. campestris* pv. *vignicola*

2 General materials and methods

2.1 Facilities and equipments

The major part of the experiments was conducted at the Benin station of the International Institute of Tropical Agriculture (IITA) in Abomey-Calavi, Benin, while complementary studies were carried out in the laboratories of the Institute of Plant Diseases and Plant Protection IPP), University of Hanover, and at the Institute of Animal Ecology, School of Veterinary Medicine, Hanover, Germany.

2.2 Plant varieties

Cowpea variety Kpodji a local variety from Benin, susceptible to *Xanthomonas campestris* pv. *vignicola*, and cassava varieties Agric and Ben 86025, also local varieties from Benin, susceptible to *X. campestris* pv. *manihotis*, were used.

2.3 Bacteria

Xanthomonas campestris pv. *manihotis* (Bondar, 1912) (Dye, 1978) strain O_7 from the Göttingen Sammlung Phytopathogener Bakterien, Göttingen, Germany (GSPB 2511) was isolated from infected cassava plants at the IITA station in Onne, southern Nigeria. *Xanthomonas campestris* pv. *vignicola* (Burkholder, 1944; Dye, 1978) strain 16g (GSPB 2509) was isolated from naturally infected cowpea leaves with typical bacterial blight symptoms collected from experimental fields at IITA in Benin. For both strains, spontaneous mutant strains resistant to rifampicin and streptomycin were used (Fanou, 1999; Sikirou, 1999). The resistance against antibiotics facilitates selective isolation of the target strains on antibiotics-containing agar medium.

2.3 Insects

Detailed vector studies focused on *Z. variegatus*. However, the following insects species were additionally collected from a cowpea field in Benin for detection of *X. campestris* pv. *vignicola*: *Oedaleus spp.* Fieber (Orth.: Acrididae), *Apis mellifera* L. (Hym.: Apiidae), *Ootheca mutabilis* Sahl (Col.: Chrisomelidae), *Milabris* spp. (Col.: Meloidae), *Exochomus troberti* Mulsant (Col.: Coccinelidae), *Pyrgomorpha cognata* Krauss and *Chrotogonus senegalensis* Krauss (both Orth.: Acrididae).

2.4 Field trials

The cassava field trial consisted of four plots (termed A, B, C and D). Each plot (3 x 3 m) consisted of 32 plants each. Sufficient distance was kept between the plots to avoid transfer of *X. campestris* pv. *manihotis*.

An experimental cowpea plot (6 x 6 m) was established in Save (the Zou department of Central Benin) for collection of insects from *X. campestris* pv. *vignicola*-infected plants. The plot was surrounded by a maize field.

2.5 Planting and maintenance

Mature stem cuttings of 20 cm length deriving from apparently healthy cassava plants were planted after a rainfall on flat ground at a spacing of 1 x 1 m during the vegetation period of 1997 at the IITA station in Benin situated in the forest savanna transition zone, where the grasshopper *Z. variegatus*. The plots were covered with a net to keep the released insects, and the ground was covered with a plastic sheet to facilitate the subsequent collection of insect faeces (Fig. 2.1). No fertiliser was used.

Cowpea seeds were sown at 4 seeds per hill at a spacing of 60 x 60 cm. Two weeks after sowing, the seedlings were reduced to 1 plant per hill. No fertiliser was used during the experiments. Weeding by hoes was carried out when necessary.

2.6 Greenhouse trials

Cassava stem cuttings were planted and cowpea seeds sown in plastic pots of 16 cm diameter, filled with field soil. The temperature in the greenhouse ranged from 25 to 30°C, relative humidity was 65-95 % relative humidity (RH). The plants were regularly watered.

2.7 Collection of *Zonocerus variegatus* and feeding

In the departments of Oueme, Mono and Zou of Benin, larvae and adults of *Z. variegatus* were collected from plants of *Chromolena odorata* King & Robinson (Asteracae), *Manihot esculenta* Crantz (Euphorbiacae), *Vigna unguiculata* (L.) Walp (Fabacae), *Ananas comosus* L. (Bromeliacae) and *Panicum maximum* Jacq (Poaceae) via sweep netting and subsequently kept in cages (Fig. 2.2). The collected insects were fed on *Vernonia amygdalina* Del. (Asteracae) leaves, a plant species possessing a known antibiotic activity (Huffman, 1997; Mintesnot and Mogessie, 1999) for two weeks in order to eliminate internal micro-organisms, which may make isolation of the target pathogen difficult.

Fig. 2.1: Experimental plot with cassava plants

Fig. 2.2: Cage for keeping *Z. variegatus*

2.8 Culture media

Nutrient Glucose Agar (NGA) (A. Mavridis, University of Göttingen, pers. comm.) was used for the isolation of pathogens and for multiplication of bacteria for inoculation. The composition of NGA was the following:

Nutrient broth	8g
Yeast extract	3g
Glucose monohydrate	11g
Bacto Agar	14g
Distilled water	ad 1000 ml

pH 7.2

The NGA was autoclaved and cooled to 45°C. Then rifampicin (100 mg dissolved in 10 ml of 100% methanol), streptomycin (100 mg dissolved in 10 ml distilled water) and cycloheximide (250 mg dissolved in 1 ml of 12.5% methanol) were filter-sterilised and added to the medium.

Additionally, the semi-selective medium (SSM) for *X. campestris* pv. *vignicola* (Khatri-Chhetri et al., 1998) was used to isolate the pathogen from naturally contaminated insects. The composition of SSM was the following:

K_2HPO_4	0.8g
KH_2PO_4	0.8g
$MgSO_4 \times 7H_2O$	0.3g
Boric acid	0.2g

NH_4Cl	1g
Distilled water	ad 1000 ml

pH 7.2

After autoclaving and cooling to 45°C, 10 mg sterile- filtered solution of cefazoline and 50 ml of a sterile-filtered mixture of 10 g D-cellobiose, 1.0 g D-methionine and 0.2 g cycloheximide, dissolved by heating to 45-50°C, were added.

2.9 Statistical analysis

The percentage of disease transmission was determined by calculating the number of infected plants per number of exposed plants to the disease. Moreover, disease incidence was presented as percentage of infected plants in the plot.

The survival of *X. campestris* pv. *manihotis* through the insect passage, and the effect of the pathogen on *Zonocerus variegatu* were determined by subjecting the data to statistical analyses. Prior to analyses data uniformity and variance homogeneity were checked. T-test was used to compare the treatments. In all comparisons a significance level of $\alpha = 5\%$ was used.

3 Detection of *X. campestris* pv. *manihotis* and *X. campestris* pv. *vignicola* in/on insects and in faeces

3.1 Introduction

Cassava and cowpea, two important staple crops in many countries in the tropics, are seriously affected by cassava bacterial blight (CBB), caused by *X. campestris* pv. *manihotis* (Bondar, 1912), and cowpea bacterial blight (CoBB), caused by *X. campestris* pv. *vignicola* (Burkholder, 1944) Dye respectively. Symptoms of both diseases, which appear generally during the rainy season, are sometimes associated with feeding of herbivorous insects (Daniel et al., 1980).

Insects are known to be vectors of plant pathogenic bacteria, (e.g. Waite, 1892 cited by Purcell, 1982, Buddenhagen and Elsasser, 1962; Goldberg and Stanghellini, 1999; Damon, 2000) such as *Erwinia amylovora* (Burr.) (winslow et al.). Flower-visiting insects in bacterial blight-infected orchards were often contaminated with *E. amylovora* (Schroth et al., 1974; Harrison et al., 1980). The relationship between *Z. variegatus* L. (Orth.: Acridiae) and cassava is well known (Bernays et al., 1977; Bani, 1990a). The preference of *Z. variegatus* for CBB-infected plants has been previously reported (Bani, 1990b; Modder, 1994). In addition, *Z. variegatus* was found in bacterial blight-infected fields, and were contaminated by *X. campestris pv. manihotis* (Terry, 1978; Daniel et al., 1980). However, to date no detailed studies on the quantification of viable bacteria in/on the insects have been carried out. Also, the distribution and survival of the pathogens in/on the insects vectors and the localisation of the bacteria within the gut of the insect have not been clearly defined.

For developing efficient control measures against bacterial diseases, a rapid and accurate detection of the causal organism is necessary as well as an understanding of the epidemiology and the disease cycle, including the identification of the inoculum sources and the ways of

dissemination of the pathogen. An important factor in the epidemiology can be the interaction with insects. It is not known, how long and where the bacteria can be localised in the insect body. Various methods such as ElISA and immunofluorescence have been compared to determine for instance the association of *E. tracheiphila* (Smith) in *Acalymma vittata* F. (Col.: Chrysomelidae) (Garcia-Salazar et al., 2000a). In addition, immunofluorescence has been used to detect *X. campestris* pv. *manihotis* from insect species collected from diseased cassava plants (Daniel et al., 1980). However, detailed information on the localisation of the pathogens on/in the insect body and the number of bacteria surviving the insect passage through the salivary glands and the intestines of the host insect are lacking. Biochemical and physiological tests which are routinely used to identify plant pathogenic bacteria (Breed et al., 1957; Buchanan and Gibbons, 1974 both cited by Schaad, 1979) are not entirely satisfactory and not well studied for detection in insects. Viable bacteria can be detected by isolation on agar, while the indirect immunofluorescence procedure can be used to localise the bacteria in the insect gut tissue.

Therefore, our objective was to quantify and localise *X. campestris pv. manihotis* and *X. campestris pv. vignicola* in/on *Z. variegatus* and other potential insect vectors. In the present study, indirect immunofluorescence, using fluorescent dye Cy3, was conjugated to a secondary antibody that is specific for the primary antibody (De Boer, 1990) and was used to localise *X. campestris pv. manihotis* antigen in the insects. To confirm, that bacteria are viable, isolation on agar was used.

3.2 Materials and methods

1.1.1 3.2.1 Preparation of inoculum

X. campestris pv. *manihotis* and *X. campestris* pv. *vignicola*, conserved on agar slant tubes containing glucose yeast extract agar (GYCA) (glucose 5g/l, yeast 5g/l, $CaCO_3$

10g/l, agar 15g/l) (Dye, 1962), at 16°C were purified by streaking a loopful of inoculum to obtain single colonies on Nutrient Glucose Agar (NGA) (nutrient broth 8g, yeast extract 3g, glucose monohydrate 11g, Bacto agar 14g). The agar plates were incubated at 30°C (Lozano and Sequeira, 1974) for 48 h in an incubator (Heraeus BK 600). A typical single colony was streaked onto many agar plates and incubated for another 48h at 30°C. Then, the bacteria were scraped and washed off with sterile 0.01 M $MgSO_4$ solution. The suspension was adjusted with a spectrophotometer (Spectronic 20, Bausch and Lomb, Cat. No. 33-29-59, USA) to an optical density of 0.06 $OD_{660\ nm}$ to give a concentration of ca. 10^8 cells/ml.

3.2.1 Plant inoculation in the greenhouse and in the fields

In greenhouse trials, the suspension was carefully infiltrated into the stomata of the abaxial surface of the first two trifoliates until water-soaked spots appeared, by means of a glass atomizer (Hokawat and Rudolph, 1991). Changing from one strain to another, the glass atomizer was washed with 70% ethanol and water. Control plants were inoculated with water. After fifteen min, the water had evaporated from the water-soaked areas and leaves regained their original colour.

In the field, one month old cassava plants of plot A (see under 2.5) and one three weeks old cowpea plants were inoculated by spraying a suspension of 10^7 cells/ml *X. campestris* pv. *manihotis* strain O_7 (GSPB 2511) and *X. campestris* pv. *vignicola* strain 16g (GSPB 2509), respectively, with a motorised sprayer (model "Solo 422", Germany) on the lower surface of the leaves. To prepare the suspension, 1 l of the stock suspension (10^8 cells/ml) was diluted with 9 l of tap water in the sprayer tank and a few drops of Tween 80 were added to increase adherence of bacteria to the leaves. The inoculation was carried out after 5 p.m. A second inoculation followed two weeks later at the same of the day.

3.2.3 Release of insects

For desinfection purposes, *Z. variegatus* were first fed on *Vernonia amigdalina* (Del.) (Asteraceae) for 7 days. Thereafter, grashoppers were released in the A plot of the experimental cassava field which had developed typical CBB symptoms following spraying with *X. campestris* pv. *manihotis.* The insects were given one week of access period to acquire the pathogens. The procedure was repeated three times.

3.2.4 Detection of *X. campestris* pv. *manihotis* in/on *Z. variegatus* by pathogen isolation

Before each transfer of *Z.variegatus* from plot A to plot B (for details refer to 2.5), the number of *X. campestris* pv. *manihotis* on the mandibles, legs, in the alimentary canal and in the faeces of 15 randomly selected insects was quantified. In an additional trial, insects were fed only with infected cassava leaves in a cage before detection of *X. campestris* pv. *manihotis*. Because of detection insensitivity, insects were assayed in groups of 5 to maximise the probability of detection. The 15 insects were divided in three groups of five insects each. For detection, the method developed by Daniel et al. (1980) was used. The mandibles, legs, the intestinal canal and the faeces were crushed in 0.01 M $MgSO_4$ solution. The liquid was filtered and centrifuged (Heraeus BK 600, Germany) at 5.21x g for 15 min. The supernatant was decanted and the pellet was suspended in 5 ml of 0.01 M $MgSO_4$. Serial dilutions were prepared and 0.1 ml of each dilution was streaked on the surface of two NGA plates or on the SSM (Khatri-Chhetri et al., 1998). The SSM was used in the trial for detection of bacteria in insects collected from naturally infected fields. After incubation for 24-48 h at 30°C, bacterial colonies were counted.

3.2.5 Detection of *X. campestris* pv. *vignicola* in/on insects collected from a bacterial blight-infected cowpea field by isolation

The collection of insects in the cowpea field started when blight symptoms had developed on the leaves after artificial inoculation of the pathogens (for details refer to 3.2.2). Every two weeks, ten insects were collected and placed individually in sterile test tubes containing 5 ml of 0.01 M $MgSO_4$. The wash-water of each tube was used for isolation of *X. campestris* pv. *vignicola* from the exterior parts of the insects on NGA supplemented with rifampicin and streptomycin. Detection on/in the different organs was performed (for details refer 3.2.4).

3.2.6 Cross reaction of antiserum against *X. campestris* pv. *manihotis* tested by agglutination

We followed the method developed by Schaad et al. (1990). Ten antisera for *X. campestris pv. manihotis* were obtained from the institute of Plant Pathology and Plant Protection, University of Göttingen. All 10 antisera were tested with *X. campestris* pv. *manihotis* strain antigen (homologous reaction), though not with the strain used for immunofluorescence:

Antisera	Antigen of *Xcm*	GSPB	Rabbit	Bleeding
A	*Xcm*15	2358	75	3rd
B	*Xcm*15		75	4th
C	*Xcm*15		75	5th
D	*Xcm*15		86	3rd
E	*Xcm*15		86	4th
F	*Xcm*15		86	5th
G	O_3	2755	76	3rd
H	O_3		76	4th
J	1312	-	13	2nd
K	1313	-	13	3rd

After growth for 48 h on NGA, a thick bacterial suspension was prepared from single colonies in 25 ml sterile saline in 50 ml falcon tubes and centrifuged (5,000 x g, 15 min) to wash off the slime of extra-cellular polysaccharides. The pellet was suspended in 25 ml saline and slime was washed off again as before. The pellet was suspended in 10 ml saline (0.85% NaCl) and the OD_{660} adjusted to 0.5 – 0.6 by a spectrophotometer (Spectronic 20, Bausch and Lomb, cat. No. 33-29-59, USA). Plastic Petri dishes were labelled and 10 µl antisera from undiluted and nine dilution levels (1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280, 1/2560) were mixed with 10 µl bacterial suspension or antiserum. For control, 10 µl saline was mixed with 10 µl bacterial suspension or antiserum. The suspensions were mixed with tooth sticks. The Petri dishes were put into a small plastic box lined with wet papers, covered properly to avoid evaporation and incubated at 37°C. Agglutination in form of white clumps was observed 16 h after incubation under a Binocular Zeiss(x25 and x40).

3.2.7 Cross reaction tested by immunofluorescence

The two antisera (1312 J and 1313 K) directed against *X. campestris* pv. *manihotis* were tested for cross- reaction with other bacterial species/genera by immunofluorescence. Generally, the same method as described in detail under 3.2.8 was followed. However, some specific steps were added.

Preparation: After growth for 48 h on NGA, a thick bacterial suspension was prepared from a single colony of each of the following seven different strains, i.e. *Erwinia carotovora* (Jones, Winslow et al.) GSPB1405, *E. carotovora* GSPB 1404, *Escherichia coli* OP50, *Pseudomonas tomato* GSPB 493, *P. apii* GSPB 2545, *X. campestris pv. manihotis* GSPB 2711, *X. campestris pv. vignicola* GSPB 2509. One droplet of each bacterial suspension was deposed on an individual slide and smeared over the surface.

Fixation: Bacteria were fixed on slides (i) by warming (70°C for 10 min), or (ii) by drying at room temperature, submerging in methanol for 1 min and subsequent drying at room temperature.

Permeabilisation: The slides were treated with PTX (0.1% Triton in PBS) three times for 5 min. Blocking and immunostaining were conducted as described in 3.2.8.

3.2.8 Detection of *X. campestris* pv. *manihotis* in insects by immunocyto-chemistry

This study was carried out in collaboration with the Institute of Animal Ecology, research group Professor Bicker, School of Veterinary Medicine in Hanover. Indirect immunocytochemistry (Haase, 2000; Hähnlein et al., 1996) was used. *Zonocerus variegatus* collected from a cassava field in Benin were fed on *V. amygdalina* leaves for one week. Then 20 insects were fed on *X. campestris* pv. *manihotis*-infected cassava leaves, and 20 insects on healthy cassava leaves for one week. For transport from Benin to Hanover, insects were conserved in 70% ethanol individually in plastic tubes.

Tissue preparation: From contaminated and non-contaminated *Z. variegatus*, heads were teased away from the body while immersed in 70% alcohol and dissected. The whole gut was obtained from the decapitated grasshoppers by gripping the lower end of the abdomen with forceps and gently pulling it and the viscera away from the body. The removed tissues were cut into pieces of 2 cm^2 with a thickness of 5 mm and separated into foregut and hindgut. Then the guts were washed in 100, 96, 85, 70 and 50% alcohol and transferred into phosphate-buffered saline PBS (8.175g/l NaCl; 1.35 g/l $NaHPO_4$; 0.22 g/l NaH_2PO_4) at pH 7.4.

Fixation: After alcohol treatment and transfer to PBS, cut tissues were immediately immersed in 4% paraformaldehyde [(PFA) in 0.1 M PBS, pH 7.4], and incubated overnight at 4°C to allow penetration of paraformaldehyde.

Paraffin embedding: Fixed samples were immersed in PBS three times for 20 min each to remove paraformaldehyde, followed by dehydration in 50, 70, 85, 96 and 100% alcohol 30 min. each.

Infiltration with molten paraffin: After two times immersion of samples in Xylol, the dehydrated samples were infiltrated in 1:1 (vol: vol) paraffin-xylen for 30 min in a vacuum oven at 56°C. Infiltration of tissue with molten paraffin (Rotiplast) (4 changes) in paraplast-X-TRA (Oxford Labware, St. Louis, MO, USA) followed quickly to avoid prolonged exposure of tissue to elevated temperature. Embedded tissues were refrigerated at 4°C until sectioned.

Sectioning: Tissue blocks were sectioned in a Leica 2035 rotary microtome (Leica Inc., Wetzlar, Germany), 10µm diameter (American Optical, Buffalo, NY, USA) and kept in water and subsequently mounted on poly-D-lysine coated slides. Sections were attached to the slide

to prevent tissue loss during subsequent incubations and washes by heating at 40° C for 24 hours.

De-paraffinisation: The sections were deparaffinised and rehydrated in order to completely remove embedding material. Slides with sections were immersed in Xylol two times for 5 min, followed by immersion in decreasing alcohol concentrations 100, 96, 85, 70 and 50% for 5 min each. Then slides were immersed in PBS three times for 5 min each, followed by one treatment in PTX (0.1% TritonX100 in PBS). The slides were dried around the tissue sections with an absorbent laboratory tissue.

Blocking: To block unspecific binding sites, the slides were treated with 3% normal goat serum (NGS) in PBT (0.5% Triton in PBS) for 2 h at room temperature.

Immunostaining:

Coupling with primary antibodies: Two undiluted polyclonal, monospecifc antisera (LPS 1313 K, LPS 1312 J) produced against lipopolysaccharides of *X. campestris* pv. *manihotis* (Rabenstein, Bundesanstalt für Züchtungforschung an Kulturpflanzen, Institut für Pathogen-diagnostik, Aschersleben, Germany, 1997) extracted from the second blood sample of immunised rabbits were diluted 1:500 in PTX. The slides were covered with antisera and incubated over night at 4° C in a closed incubation chamber.

Washing: Excess antiserum was removed from the tissue with a gentle steam of 0.1% PTX, three times for 10 min each.

Coupling with secondary antibodies: Excess PTX was removed from the slide with an absorbent wipe. Then slides were recovered with the second antibody (biotinylated goat anti-rabbit igG, Vector) diluted 1:250 in 1% PTX containing 5% NGS/Natrium Azid and incubated in an incubation chamber at room temperature for one hour.

Washing: Excess antiserum was rinsed from the tissue with 0.1% PTX two times for 10 min, and once with PTW (0.1% Tween 20 in PBS for 10 min) to remove unbound secondary antibodies.

Visualization: To visualize the antibody bound to the bacteria, slides were covered with the fluorescence dye Cy3 conjugated to Streptavidin (SIGMA) 1:250 diluted in PTW, and incubated for 1 h at room temperature.

Washing: Samples were washed again in PTW three times for 10 min.

Mounting and microscopy: The excess washing solution was drained and removed from the slide around the tissue. Then a drop of glycerin was placed on the slide covered with a coverslip. Tissue staining was observed under a darkfield microscope (Zeiss/Axiovert 25, Germany) equipped with filters (BP 485, FT 510, LP 520) appropriate for the fluorescence dye. A light source HB 050/AC with a wavelength 365-400 providing adequate illumination in the near ultraviolet range (Epi-illumination) and Neofluar objectives 10x and 20x were used. The camera RICOH KR-IOM equipped with the film KODAK 200 ASA served for documentation.

3.3 Results

3.3.1 Detection of *X. campestris* pv. *manihotis* in/on insects

Zonocerus variegatus were fed on cassava plants infected with *X. campestris* pv. *manihotis* strain GSPB 2511 marked with resistance against two antibiotics in plot A. After one week, the insects were transferred to healthy plants (plot B). Before each transfer from plot A to plot B, 15 insects were analysed for carrying the pathogen. The transfer was carried out four times. The pathogen was detected in all the analysed organs, e.g. on the mandibles, legs, in the intestines and in the faeces of the insects collected from plot A (Table 3.1). The faeces lodged

more bacteria than the organs of *Z. variegatus.* In each group of 15 insects, the pathogen was detected on the locomotion organs of *Z. variegatus*. When the insects were fed only on infected leaves in the greenhouse, the pathogen was found in higher number on all the organs and in the faeces compared to the insects which had the possibility to feed also on healthy plants in the field. In both cases, the number of bacteria per organ varied in decreasing order: faeces > intestines > legs > mandibles.

Table 3.1: Detection of *X. campestris* pv. *manihotis* (*Xcm*) in/on grasshoppers fed on bacterial blight-infected cassava plants

		Fed on infected & non-infected leaves (field)						Fed on infected leaves only (greenhouse)	
		Trial 1		Trial 2		Trial 3		Trial 4	
Organs	Replication	Cfu/insect[a]	Mean	Cfu/insect	Mean	Cfu/insect	Mean	Cfu/insect	Mean
Mandibles	A[b]	0		0		0		425 ± 2	
	B	0	0	0	3	0	0	375 ± 5	367
	C	0		10 ± 1		0		300 ± 3	
Legs	A	10 ± 2[c]		0		0		375 ± 5	
	B	0	3	10 ± 2	65	0	3	500 ± 6	622
	C	0		185 ± 5		10 ± 2		990 ± 10	
Intestine	A	5 ± 1		5 ± 0		0		925 ± 10	
	B	0	2	0	22	0	0	400 ± 5	790
	C	0		60 ± 2		0		1045 ± 10	
Faeces	A	+[d]		++		+++		++++	
	B	+	+	++	++	+++	+++	++++	++++
	C	+		++		+++		++++	

[a] Cfu = Colony forming units
[b] Five insects per replication
[c] Mean value with standard error
[d] + to ++++ increasing, uncountable number of cfu

3.3.2 Detection of *X. campestris* pv. *vignicola* in/on insects

Eight insect species were collected in a cowpea field infected by *X. campestris* pv. *vignicola*, and subsequently controlled for carrying the pathogen. *X. campestris* pv. *vignicola* was isolated from seven insect species (Table 3.2). The pathogen was detected on all exterior parts of the insects, and also from the intestines and the faeces of *Z. variegatus* L. (Orth.: Acrididae), *Oedaleus* spp. Fieber (Orth.: Acrididae), *Pyrgomorpha cognata* Krauss (Orth.: Acrididae). In detail, the pathogen was detected on the exterior surface, mandibles, legs, and faeces of *Oedaleus* spp. on the exterior surface of *Apis mellifera* L. (Hym.: Apiidae), on the exterior and interior organs of *Ootheca mutabilis* Sahl. (Col.: Chrisomelidae), *Milabris* spp. F. (Col.: Meloidae) and *Exochomus troberti* Mulsant (Col.: Coccinelidae) and on the exterior organs and in the faeces of *Pyrgomorpha cognata*, while the pathogen was not detected on or in *C. senegalensis* Krauss (Orth.: Acrididae).

Table 3.2: Detection of *X. campestris* pv. *vignicola* on/in different organs of insects collected in a bacterial blight-infected cowpea field

Species	Cfu/insects[a]					
	Exterior	Intestines	Faeces	Mandibles	Legs	Total
Z. variegatus	$5.4 \times 10^2 \pm 1.0 \times 10^2$	$2.1 \times 10^2 \pm 1.6 \times 10^2$	$2.0 \times 10^2 \pm 0.5 \times 10^2$	$2.8 \times 10^1 \pm 0.2 \times 10^1$	$5.4 \times 10^1 \pm 0.6 \times 10^1$	1.0×10^3
Oedaleus spp.	$4.3 \times 10^4 \pm 0.2 \times 10^4$	0	$7.8 \times 10^2 \pm 1.0 \times 10^2$	$8 \times 10^3 \pm 1.6 \times 10^3$	$2.5 \times 10^3 \pm 0.8 \times 10^3$	5.4×10^4
P. cognata	$2.2 \times 10^3 \pm 0.1 \times 10^3$	$7.8 \times 10^2 \pm 1.2 \times 10^2$	$7.5 \times 10^1 \pm 0.2 \times 10^1$	0	0	3.1×10^3
O. mutabilis	$1.8 \times 10^2 \pm 3 \times 10^2$	$5.6 \times 10^1 \pm 1.0 \times 10^1$	Ne[b]	Ne	Ne	2.4×10^2
Milabris spp.	$7.5 \times 10^3 \pm 2.5 \times 10^3$	$8.6 \times 10^1 \pm 0.4 \times 10^1$	0	$1.0 \times 10^2 \pm 0.4 \times 10^2$	0	7.7×10^3
Exochromus troberti	$4.2 \times 10^1 \pm 0.2 \times 10^1$	$1.4 \times 10^3 \pm 0.8 \times 10^3$	Ne	Ne	Ne	1.4×10^3
Apis mellifera	$1.0 \times 10^4 \pm 0.8 \times 10^4$	0	0	0	0	1.0×10^4
C. senegalensis	0	0	0	0	0	0

[a] Cfu = Colony forming units; data presented as mean values ± SE
[b] Ne = Not examined

3.3.3 Detection of *X. campestris* pv. *manihotis* in *Z. variegatus* by immuno-fluorescence microscopy

3.3.3.1 Test of antisera against *X. campestris* pv. *manihotis* for titer and cross reaction by agglutination

A positive reaction was expressed by formation of white, big clumps drifting in the suspension (Fig. 3.1), while a clear to cloudy suspension or white crystals at the bottom of the suspension were recorded as negative reaction. Antisera obtained from the first and second blood samples were used. The agglutination of *X. campestris* pv. *manihotis* cells was negative with the antisera A (7513), B (7514), C (7515), D (8613), E (8614), F (8615), G (7613), H (7614) and positive with J (1321) and K (1313) with a titer of 1/40 (Table 3.3), while the reaction of the antisera A, B, G, J and K was positive with *X. campestris* pv. *vignicola* strains GSPB 2509 with a titer of 1/40 for J and K; 1/80 for A, B and 1/20 for G (Table 3.4).

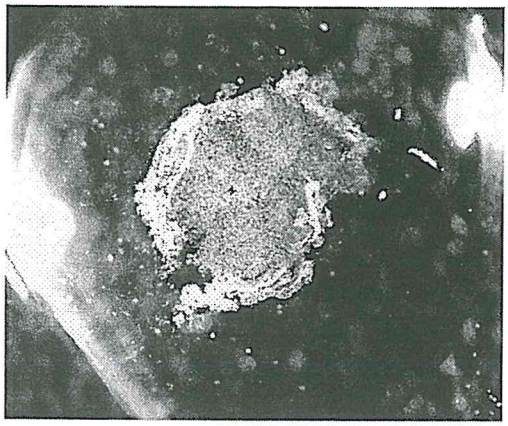

Fig. 3.1: Cross reaction tested by agglutination with cells of *X. campestris* pv. *manihotis* strain GSPB 2511

[Positive reaction: agglutination of a polyclonal monospecific antiserum J (dilution 1/2560) with *X. campestris* pv. *manihotis* strain GSPB 2511 (x 40)]

Table 3.3: Determination of titer of ten antisera against *X. campestris* pv. *manihotis* by agglutination with cells of *X. campestris pv. manihotis* strain GSPB 2511

	Agglutination of *X. campestris pv. manihotis* with dilutions of 10 antisera									
Dilution[a]	A[b]	B	C	D	E	F	G	H	J	K
1/10	-	-[c]	-	-	-	-	-	-	+++	++
1/20	-	-	-	-	-	-	-	-	++	+
1/40	-	-	-	-	-	-	-	-	+-	+-
1/80	-	-	-	-	-	-	-	-	-	-
1/160	-	-	-	-	-	-	-	-	-	-
1/320	-	-	-	-	-	-	-	-	-	-
1/640	-	-	-	-	-	-	-	-	-	-
1/1280	-	-	-	-	-	-	-	-	-	-
1/2560	-	-	-	-	-	-	-	-	-	-

[a] No reaction occurred in control samples of bacteria with saline, and saline with serum
[b]Antisera A = 7513, B = 7514, C = 7515, D = 8613, E = 8614, F = 8615, G =7613, H = 7614, J =11321 and K = 11313
[c]+++ = very good agglutination
++ = good agglutination
+ = weak agglutination
+- = very weak agglutination
- no agglutination

Table 3.4: Determination of cross-reaction of ten antisera against *X. campestris* pv. *manihotis* by agglutination with cells of *X. campestris* pv. *vignicola* strain GSPB 2509

	Agglutination with dilutions of 10 antisera									
Dilution[a]	A[b]	B	C	D	E	F	G	H	J	K
1/10	++[d]	++	-	-	-	-	+	-	++	++
1/20	+	+	-	-	-	-	+-	-	+	+
1/40	+	+	-	-	-	-	-	-	+-	+-
1/80	+-	+-	-	-	-	-	-	-	-	-
1/160	-	-	-	-	-	-	-	-	-	-
1/320	-	-	-	-	-	-	-	-	-	-
1/640	-	-	-	-	-	-	-	-	-	-
1/1280	-	-	-	-	-	-	-	-	-	-
1/2560	-	-	-	-	-	-	-	-	-	-

[a] A very weak agglutination (+-) was observed in all dilutions of antisera C, D, E, F, H and an auto-agglutination of cells observed in some control samples with saline
[b]Antisera A = 7513, B = 7514, C = 7515, D = 8613, E = 8614, F = 8615, G = 7613, H = 7614, J = 11321 and K = 11313
[c]+++ = very good agglutination
++ = good agglutination
+ = weak agglutination
+- = very weak agglutination
- no agglutination

3.3.3.2 Cross reaction of antiserum against *X. campestris* pv. *manihotis* tested by immunofluorescence

In the cross-reaction test of the antiserum against *X. campestris* pv. *manihotis* (1:500) with the bacterial genera/species/pathovar *Escherischia coli*, *Erwini carotovora* 1405 (Jones, Winslow et al.), *E. carotovora* 1404, *Pseudomonas tomato*, *P. apii* (Jagger), and *X. campestris* pv. *vignicola*, fluorescence was only observed with *X. campestris* pv. *manihotis*, but not with the other bacteria (Table 3.5), confirming the specificity of the antiserum for *X. campestris* pv. *manihotis*.

Table 3.5: Cross-reactions of six bacterial genera/species/pathovar tested by fluorescence microscopy, using the polyclonal, monospecific antisera 1313 K and 1312 J against *X. campestris* pv. *manihotis*

Species	Fluorescence
E. coli[a]	-[b]
E. carotovora[c] 1405	-
E. carotovora[c] 1404	-
P. tomato[d]	-
P. apii[d]	-
X. campestris pv.*vignicola*	-
X. campestris pv. *manihotis*	+

[a] *Escherischia,* [b] + positive reaction, - negative reaction,
[c] *Erwinia,,* [d] *Pseudomonas*

3.3.3.3 Localisation of *X. campestris* pv. *manihotis* in *Z. variegatus* tissue

A strong staining of *X. campestris* pv. *manihotis* cells was observed in gut tissues of *Z. variegatus* which had been fed on bacterial blight-infected cassava leaves. The pathogen was localised in the foregut and hindgut of *Z. variegatus*, indicating that the bacteria were present

throughout the alimentary canal. In all 20 samples with infected tissues, the presence of the bacteria was verified (Fig. 3.2). There was no clear difference in the number of bacteria in the fore and hindguts, though a tendency of higher bacterial numbers in the foregut was recorded. Bacteria occurred either single or in clumps within a membrane in the interior or near the periphery of the tissue. Clusters of bacteria were more frequently found in the fore- than in the hindgut. The antigen also associated in folds and winkles of the hindgut. The gut samples of *Z. variegatus*, which had been fed on healthy cassava leaves showed only a light staining of single few bacteria (Fig. 3.3). The number of bacteria in these tissues was much lower than the number in the tissues of insects fed on bacterial blight-infected leaves only. Some bacteria were also found outside the tissues.

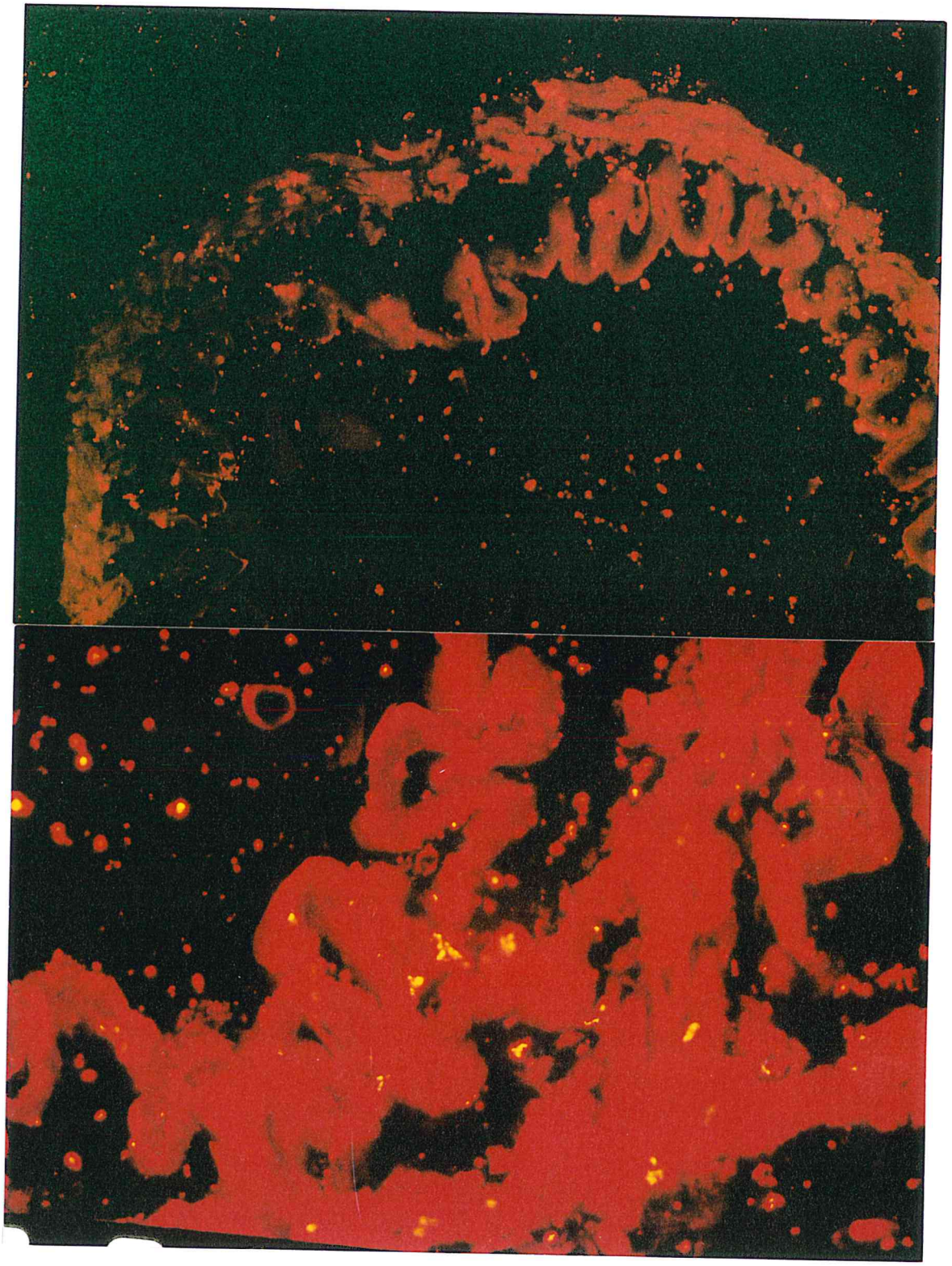

Fig. 3.2: Localisation of *X. campestris* pv. *manihotis* in infested *Z. variegatus* gut tissue

(Upper photo: Neofluar objective X 10, lower photo: Neofluar objective X 20)

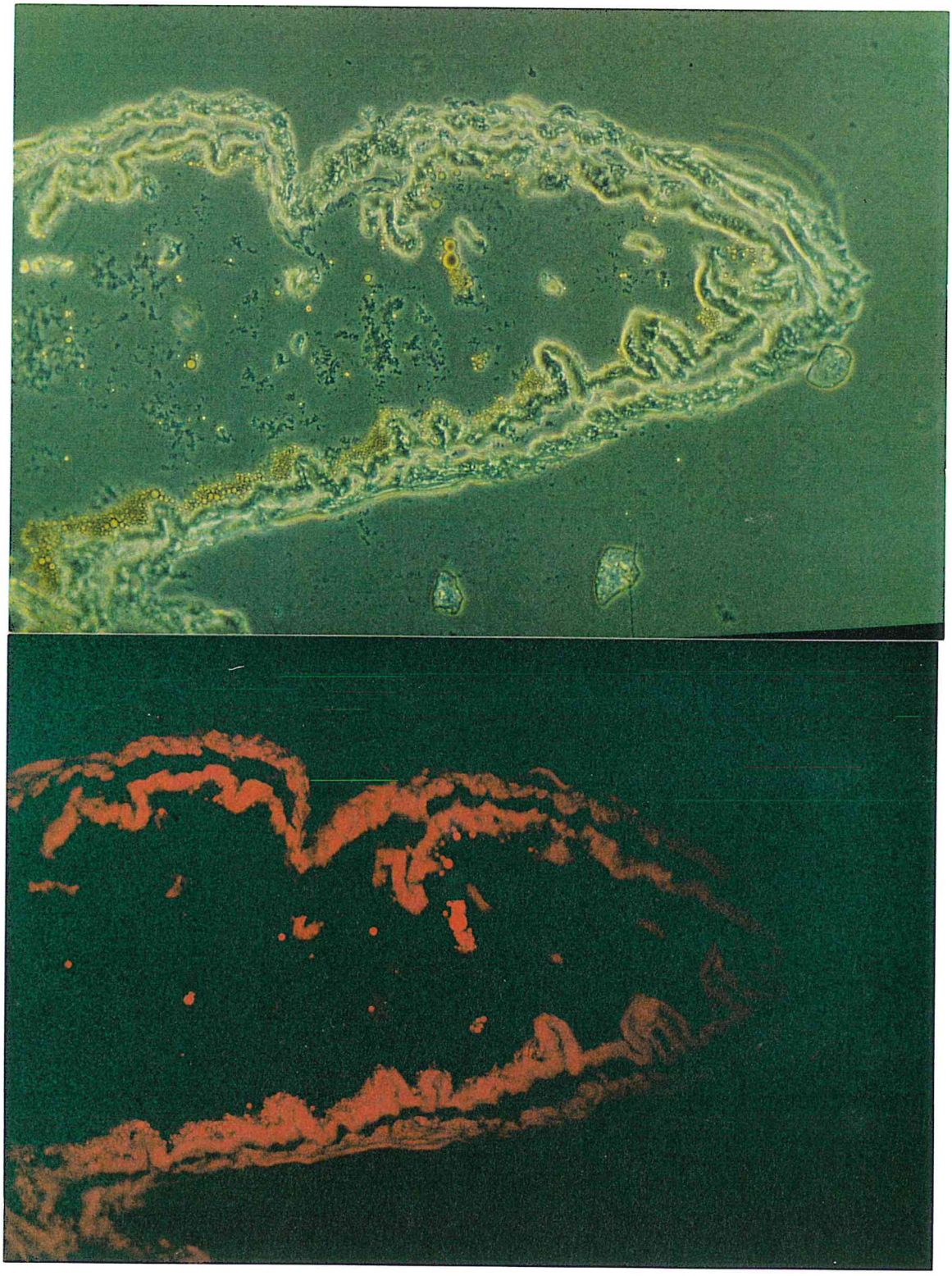

Fig. 3.3: Gut tissue of non-infested *Z. variegatus*

(Upper photo: light microscopy, lower photo: fluorescence microscopy, Neofluar objective X 10)

3.4 Discussion

The main objective of the studies was to elucidate the possible role of insects in the dissemination of cassava and cowpea bacterial blight under field conditions in Benin.

Cassava bacterial blight

Several authors had demonstrated feeding of *Z. variegatus* for cassava (e.g. Kaufmann, 1965; Terry and Goodman, 1977; Modder, 1994; Le Gall et al., 1998) suggesting that the insect might carry and possibly transmit the pathogen. Our studies confirmed that *Z. variegatus* were contaminated during their movement in a bacterial blight infected cassava field. *X. campestris* pv. *manihotis* was isolated from mandibles, legs, the alimentary canal, and in high number also from the faeces of the insect. Also Daniel et al. (1980) and Bani (1990b) demonstrated the presence of the bacteria on the insect exoskeleton (wash water), in the digestive system and faeces by isolation, though they did not quantify the contamination of the different organs. The high number of bacteria on/in the grasshoppers when feeding only with infected leaves suggested that the contamination rate depended on the inoculum quantity. However, it was not possible to use naturally contaminated field-collected insects in these studies because bacterial blight and different insects species occurred seasonally and collecting and maintaining sufficient naturally infested insects to carry out the different experiences proved difficult. The presence of *X. campestris* pv. *manihotis* in/on grasshoppers was confirmed using immunofluorescence microscopy, by which the bacteria were localised in the insect fore- and hindgut. The immunofluorescence method allows the specific detection of bacteria in situ, and the polyclonal, monospecific antisera directed against lipopolysacchrides (LPS) proved suitable for detection of *X. campestris* pv. *manihotis* in tissues of insects collected in Benin. The serological detection of *X. campestris* pv. *manihotis* also in the control groups of insects which had previously been fed for one week on *Vernonia* spp. to eliminate intestinal

insects which had previously been fed for one week on *Vernonia* spp. to eliminate intestinal bacteria, may be due to natural contamination of the field-collected insects and suggest, that *Vernonia spp.* did not provide complete eradicative antibiotic effects on *Xanthomonas campestris pv. manihotis* as previously reported in the literature for other micro-organisms (Gebreyojannes and Drassar 1985; Mintesnot and Mogessie 1999). Our results suggest that *Xanthomonas* spp. move into the salivary ducts via feeding on infected leaves. It could be shown that the bacteria survive the passage through the insects in considerable numbers although the gut and saliva are known to contain a number of potentially harmful digestive enzymes (Bani, 1990b). However, contamination of the insect tissue with *X. campestris pv. manihotis* may partly derive from the treatment of the insects and the tissue during transportation, dissection and preparation of the paraffin specimen, although our observations revealed that the major part of bacteria was found attached to and inside the tissue of the gut. The stringency of the fixation, embedding and deparaffination process to which inoculated tissues was subjected before immunostaining may reduce non-specific binding of the antisera. Nevertheless, antibody, or the substrates used for immunofluorescence can produce titer-dependent positive responses to a few other currently unidendified bacterial isolates from the alimentary canal of *Z. variegatus*. However, to assure about the specificity of the antibody, cross-reactions were done first by agglutination and then by immunofluoresence. We found a cross-reaction of the antisera directed against *X. campestris* pv. *manihotis* in agglutination tests. The cross-reaction test with immunofluorescence showed that our antibody did not produce titer-dependent positive responses to other test bacteria than *X. campestris* pv. *manihotis*. Also the titer found with immunofluorescence was much higher and a reaction was still observed at 1:5000, while in the agglutination test a titer of only 1/40 was recorded. This may be due to the high specificity of this monospecific antiserum, which reacts only with a limited number of antigens on the bacterial surface, which is not sufficient to agglutinate

fluorescence dye conjugated antiserum. According to Schaad, (1979), the titer found with agglutination tests is easily performed and rapid, but not very sensitive and inadequate for distinguishing between closely related immunogens. Thus, we judge the results of the titer and cross-reaction tests by agglutination as unreliable. The apparition of weak agglutination (see under 3.3.3.1), which was observed in all dilutions of antisera, showed that something was wrong. Obviously *X. campestris* pv. *vignicola* tends to auto-agglutinate. In our studies with insect tissues, we used **indirect** immunofluorescence which is reported to be superior over the direct method because (i) it is simpler than the direct method (although the staining requires more time), (ii) the dye is conjugated to antirabbit globulin and not directed to the antisera and to the organism to be identified, (iii) the antirabbit serum is available commercially (Schaad, 1979) and (iv) requires very little antisera.

Detection by isolation confirmed that bacteria isolated from the intestinal canal, and from the faeces were still viable. Daniel et al. (1980) also detected *X. campestris* pv. *manihotis* in 40% of the examined *Z. variegatus* by immunofluorescence. Also, the association of *E. tracheiphila* in *Acalymma vittatum* F. (Col.: Chrysomelidae) was determined by immunolocalisation and additionally by Elisa (Garcia et al., 2000a). Our immunolocalisation data suggests a higher rate of acquisition and retention within *Z. variegatus* with high transmission than reported by Wayadande and Fletcher (1995) under their caged bioassays. The detection of the pathogen for 28-34 days in the insect gut after acquisition suggests a long-term endosymbiotic associations of *X. campestris* pv. *manihotis*. Similar associations where found with other bacteria species like *Erwinia spp.* and insects (Elliott and Poos, 1934).

Since some insect samples and their guts were not in good conditions after storage and transport from Benin in 70% ethanol, for furture trials transport of whole insects should be avoided and paraffin embedding should be done immediately after collection of insects.

Since some insect samples and their guts were not in good conditions after storage and transport from Benin in 70% ethanol, for future trials transport of whole insects should be avoided and paraffin embedding should be done immediately after collection of insects.

Cowpea bacterial blight

All insects collected in bacterial blight-infected cowpea fields carried *Xanthomonas campestris pv. vignicola*. Our results corroborate earlier finding by Walter and Vakili (1977) who showed that five insect species (*Cerotoma ruficornis* Oliv. (Col.: Chrysomelidae), *Chalcodermus ebeninus* Boheman (Col.: Curculionidae), *Diaprepes abbreviata* L. (Col.: Curculionidae), *Empoasca sp* (Hem.: Cicadelina), and *Nezara viridula* L. (Hem.: Pentatomidae)) which are pests of foliage and flower of cowpea plants, acted as vectors of cowpea bacterial blight in Costa Rica. We also found that specimens of *A. mellifera* L. were contaminated with *X. campestris* pv. *vignicola*. Bees have been shown as effective vectors of *Erwinia amylovora* (Burr) (Winslow et al.), which were dispersed by the activity of honey bees from bacterial colonies in the first early opening flowers to other open flowers (Steiner, 2000).

4 Transmission of *X. campestris* pv. *manihotis* and *X. campestris* pv. *vignicola* by *Z. variegatus*

4.1 Introduction

The causal agent of bacterial blight in cassava and cowpea *X. campestris* pv. *manihotis* (Bondar, 1912) and *X. campestris* pv. *vignicola (Xcv)* (Burkholder, 1944) (Dye, 1978) respectively, have been associated with the grasshopper *Z. variegatus* (Daniel et al., 1980; M. Zandjanakou, unpubl. res.). Several plant pathogenic bacteria have been demonstrated to be transmitted by insect vectors (e.g. Harrison et al., 1980; Garcia-Salazar et al., 2000a & 2000b). In this study, the term vector is used for insect dissemination and transmission of plant pathogens. Dissemination refers to the transfer of the pathogen or the bacterial inocula by insects from diseased to healthy plants. Transmission includes both dissemination and inoculation by the insects when feeding, which result in the presence of epiphytic bacterial populations, subsequent infection and development of the disease. With few exceptions, insect plant pathogenic bacteria relationships are non-obligate and in many cases strictly accidental. Since bacterial inoculum is often present as a sticky exudate on the plant, insects, which come in contact with it, are readily contaminated and thus become potential vectors of the pathogens (Harrison et al., 1980). They may aid survival of the pathogen, by disseminating both primary and secondary inoculum of the pathogen from plant to plant (Walter and Vakili, 1977). Moreover, insects may cause wounds in the plant tissue, thereby facilitating the entry of bacteria into healthy plants and possibly helping pathogens to withstand unfavourable environmental conditions.

Studies and field observations revealed that *Z. variegatus* is a vector of many plant diseases. For instance the insect transmits okra mosaic virus (OMV) with an efficiency of 10% (Givord and Den Boher, 1980) and cowpea mosaic virus (CMV) with an efficiency of 19% (Whitney

and Gilmer, 1974). In addition, *X. campestris* pv. *manihotis* was also found in the wash water of *Gonocephalum simplex* (Fab.) (Col.: Tenebrionidae) and *Ischnotrachelus* spp. (Col.: Curculionidae), *Z. variegatus* L. and also in the alimentary canal and faeces of these three insects (Daniel et al., 1980). However, the role of insects as potential vectors for the epidemiology of these pathogens is not clear. No detailed investigations on the transmission of *X. campestris* pv. *manihotis* and *X. campestris* pv. *vignicola* by herbivorous insects has been carried out. The feeding behaviour of grasshoppers in cassava and cowpea and possible preferences of *Z. variegatus* for *X. campestris pv. manihotis*-infected cassava leaves are unknown. The exact ways of vector transmission for these diseases is not known, but vector feeding wounds seem to play a role. Therefore, in this study, the transmission of the bacteria by contaminated *Z. variegatus* and by their faeces was investigated and the inoculum level of *X. campestris* pv. *manihotis* and *X. campestris* pv. *vignicola* necessary for symptom development under environmental conditions was determined.

4.2 Materials and methods

4.2.1 Transfer of *X. campestris* pv. *manihotis*-contaminated *Z. variegatus* from plot A to plot B

After feeding in plot A (see under 2.5) for one week, 159 insects were transferred to plot B (healthy cassava plants of two months age). Out of the total of 219 released insects, 60 were used for pathogen detection. Two hundred nineteen non-contaminated *Z. variegatus* were released in control plot C, while control plot D contained healthy cassava plants without *Z. variegatus*.

4.2.2 Symptom evaluation in the field

Plants in plots B, C and D were regularly inspected for incidence of plants showing bacterial blight symptoms. Two weeks after the first release of contaminated grasshoppers in plot B, some leaves with symptoms were collected in this plot for later isolation of the pathogens. The percentage of transmission was calculated in plot B independently of the number of insects.

4.2.3 Infection of cassava plants by *X. campestris* pv. *manihotis*-contaminated faeces of *Z. variegatus*

Twenty grasshoppers collected from heavily bacterial blight-infected cassava plants in the greenhouse were kept in screening cages for collection of faeces. For transmission studies, healthy cassava leaves were scarified with a scalpel or wounded with a cork borer. The remaining leaves were left undamaged. One g of contaminated faeces were crushed in 5 ml of 0.01M $MgSO_4$, and the suspension was poured on the scars or wounds on the upper and lower leaf surfaces of the damaged and intact leaves. Plants were kept in the greenhouse (25-30°C and 65-95 RH) for two weeks. Leaves were humidified twice per day by spraying tap water. Symptom development was regularly observed.

4.2.4 *X. campestris* pv. *manihotis* inoculum level necessary for symptom development

Ten cassava plants were sprayed with two concentrations of *X. campestris* pv. *manihotis* strain GSPB 2511 (10^2 cfu/ml; 10^4 cfu/ml). The plants were regularly evaluated for symptoms expressed in four symptom classes: class 1 = no symptom, class 2 = water-soaking spots on leaves, class 3 = leaf blight, class 4 = leaf wilt, and class 5 = dieback. Control plants were sprayed with tap water.

4.2.5 Infection of cowpea plants by *X. campestris* pv. *vignicola*-contaminated *Z. variegatus*

Fifteen grasshoppers, collected from heavily bacterial blight-infected cowpea plants in screening cages in the greenhouse were transferred to healthy, four weeks-old cowpea plants for five days. Plants were kept in the greenhouse (25-30°C and 65-95 RH), and leaves were humidified twice per day by spraying tap water. Symptom development was regularly observed (for details refer to 4.2.4).

4.2.6 *X. campestris* pv. *vignicola* inoculum level necessary for symptom development

Fifteen cowpea plants were sprayed with two concentrations of *X. campestris* pv. *vignicola* strain GSPB 2509 (10^2 cfu/ml; 10^4 cfu/ml). The plants were regularly evaluated for symptoms expressed in four symptom classes: class 1 = no symptom, class 2 = water-soaking spots on leaves, class 3 = leaf blight, class 4 = leaf wilt, and class 5 = dieback.

4.3 Results

4.3.1 Transmission of *X. campestris* pv. *manihotis* by *Z. variegatus* in the field

Two weeks after the transfer of *Xanthomonas campestris pv. manihotis*-contaminated grasshoppers from plot A to plot B, a higher disease incidence was recorded in plot B than in control plots C and D (Table 4.1). Many angular leaf spots and some blight symptoms were observed on previously healthy cassava plants in plot B. The disease incidence of 18.2% recorded in the grasshopper-free control (plot D) was due to natural infection of cassava plants with bacterial blight. The marker strain GSPB 2511 could not be reisolated from symptoms on plants of plot B.

Table 4.1: Percentage of transmission and disease incidence in cassava plots with *X. campestris* pv. *manihotis*-contaminated (B) and non-contaminated (C) grasshoppers and without grasshoppers (D) eight weeks after the first transfer of contaminated insects

Plots[a]	% of transmission [b]
B	43.75
	Disease incidence
C	10.3%
D	18.2%

[a] Each plot contained 32 plants
[b] Number of plants infected per number of plants exposed

4.3.2 Transmission of *X. campestris* pv. *manihotis* by contaminated *Z. variegatus* faeces

Five days after treatment of damaged and non-damaged cassava leaves with *X. campestris* pv. *manihotis* contaminated faeces collected from insects that had previously fed on infected plants, damaged leaves showed typical angular leaf spots (Fig. 4.1), while first symptoms on undamaged leaves appeared seven days after the initial treatment (Fig. 4.2). In three repeated trials the transmission rate was 90% for the scarified and wounded leaves (Table 4.2), while

for intact leaves a transmission rate of 46.7% and 63.2% was recorded after deposing faeces on adaxial and abaxial surfaces, respectively.

Table 4.2: Symptoms of *X. campestris* pv. *manihotis* on undamaged and damaged cassava leaves after deposing contaminated faeces of *Z. variegatus* on abaxial and adaxial leaf surfaces

	Treatment[a]			
Replication	Scarifications[b]	Holes	Intact adaxial surface	Intact abaxial surface
1	10/10	10/10	2/5	2/5
2	4/5	4/5	5/5	2/6
3	4/5	4/5	0/5	2/8
Total	18/20	18/20	7/15	6/19
% transmission	90.0	90.0	46.7	63.2

[a] Number of plants infected per number of plants exposed

[b] Cassava leaves were scarified, wounded or kept intact before placing infested faeces on the leaf surfaces.

4.3.3 Determination of infectivity titer of *X. campestris* pv. *manihotis* on cassava

Seven days after spray-inoculating cassava plants with a *X. campestris* pv. *manihotis* suspension of 10^4 cfu/ml, typical water-soaking symptoms appeared (Table 4.3), which further developed into blight and wilt symptoms and in some cases into dieback. After inoculation with 10^2 cfu/ml, the first symptoms started two weeks after inoculation (Table 4.4). Not all the plants developed symptoms and for those with initial symptoms, symptom development was lower compared to the inoculation with a concentration of 10^4 cfu/ml.

Fig. 4.1: Water-soaked leaf spot symptoms on undamaged cassava leaf

Fig. 4.2: Water-soaked leaf spot symptoms on damaged cassava leaf

Table 4.3: Number of cassava plants in symptom classes after inoculation with 10^4 cfu/ml of *X. campestris* pv. *manihotis* strain GSPB 2511

Classes [a]	Number of plants with symptoms					
	7 dpi [b]	14 dpi	21 dpi	28 dpi	39 dpi	46 dpi
Class 1	7	6	1	1	1	1
Class 2	3	2	5	4	2	1
Class 3	0	2	4	5	5	2
Class 4	0	0	0	0	2	5
Class 5	0	0	0	0	0	1

[a] Class 1 = no symptoms; class 2 = water-soaked spots on leaves; class 3 = leaf blight; class 4 = leaf wilt; class 5 = dieback
[b] days post inoculation

Table 4.4: Number of cassava plants in symptom classes after inoculation with 10^2 cfu/ml of *X. campestris* pv. *manihotis* strain GSPB 2511

Classes [a]	Number of plants with symptoms					
	7 dpi [b]	14 dpi	21 dpi	28 dpi	39 dpi	46 dpi
Class 1	10	8	7	6	6	6
Class 2	0	2	3	3	2	2
Class 3	0	0	0	1	2	1
Class 4	0	0	0	0	0	1
Class 5	0	0	0	0	0	0

[a] Class 1 = no symptoms; class 2 = water-soaked spots on leaves; class 3 = leaf blight; class 4 = leaf wilt; class 5 = dieback
[b] Days post inoculation

4.3.4 Infection of cowpea plants by *X. campestris* pv. *vignicola*-contaminated *Z. variegatus*

The transfer of *X. campestris* pv. *vignicola*-contaminated *Z. variegatus* to healthy cowpea plants under greenhouse conditions did not result in the apparition of bacterial blight symptoms. However, *X. campestris* pv. *vignicola* numbers of up to 8.9×10^4 cfu/g leaves were

detected on the formally healthy leaves one week after the transfer of contaminated *Z. variegatus* from the infected cowpea leaves (Table 4.5).

Table 4.5: Epiphytic population of *X. campestris* pv. *vignicola* on cowpea leaves transmitted by contaminated *Z. variegatus* fed on infected leaves

Repetitions[a]	Cfu[b]/g[c]
1	$8.9 \times 10^4 \pm 0.5 \times 10^4$
2	$4.6 \times 10^1 \pm 1.0 \times 10^1$
3	$2.6 \times 10^1 \pm 0.5 \times 10^1$
4	0
5	0

[a] Two leaves per plant were used.
[b] Colony forming unit.
[c] Mean of two Petri dishes (± SE).

4.3.5 Determination of infectivity titer of *X. campestris* pv. *vignicola* on cowpea

Cowpea plants were sprayed with two concentrations of *X. campestris* pv. *vignicola* suspensions (i.e.10^2 and 10^4 cfu/ml) in order to determine the inoculum level necessary for symptom development under greenhouse conditions. Nineteen days after inoculating cowpea plants with 10^4 cfu/ml, typical water-soaking symptoms appeared (Table 4.6). However, no symptoms were detected on plants sprayed with 10^2 cfu/ml.

Table 4.6: Number of cowpea plants in symptom classes after inoculation with two different inoculum concentrations of *X. campestris pv. vignicola* strain GSPB 2509

Classes[b]	Number of plants with symptoms						
	10^4 cfu[a] /ml						10^2 cfu/ml
	12 dpi[c]	19 dpi	26 dpi	32 dpi	39 dpi	46 dpi	19-46 dpi
Class 1	15	9	6	3	2	2	0
Class 2	0	5	5	5	4	3	0
Class 3	0	1	4	4	4	3	0
Class 4	0	0	0	3	4	5	0
Class 5	0	0	0	0	1	2	0

[a] Colony forming unit.
[b]Class 1 = no symptoms; class 2 = water-soaked spots on leaves; class 3 = leaf blight; class 4 = leaf wilt; class 5 =dieback
[c] Days post inoculation

4.4 Discussion

Cassava bacterial blight

The objective of these studies was to demonstrate the transmission of the bacterial blight pathogens of cassava and cowpea to healthy plants by potential insects vectors in the field and under controlled conditions. Transmission of propagative plant pathogens by insect vectors is a complex process, since, theoretically, it is difficult to prove that a particular insect species cannot be able to transmit a particular pathogen under any circumstance. Environmental influences on disease development after inoculation are of direct concern in such investigations (Purcell, 1982).

The transfer of *Z. variegatus* from bacterial blight-infected cassava plant to healthy plants in the field resulted in higher transmission rate of bacterial blight symptoms compared to the control. This transmission rate was recorded after a long acquisition period (one week). Nevertheless, our attempt to reisolate the antibiotics-resistant marker strain from symptom-carrying plants failed. This may be explained by the progressive loss of antibiotic resistance by bacteria under natural conditions where this trait is no advantage for survival. Also Bani, (1990b) found that all the cassava plants on which contaminated *Z. variegatus* were fed, later developed bacterial symptoms under laboratory conditions (t = 27°C, RH = 80%).

The transmission of the disease by pathogen-contaminated faeces of *Z. variegatus* to wounded, scarified and intact healthy cassava leaves could be demonstrated. For many pathogens, especially for plant pathogenic bacteria, of which many are incapable of directly penetrating plants, wounds are either the most frequent or second avenues of entry besides stomata. However, Bani (1990b) did not observed symptoms after pathogen contaminated-faeces of *Z. variegatus* were deposited on intact cassava leaves. These conflicting results may

be due to the different environmental conditions in Bani (1990b) trial in comparison to our experiments. The lower percentage of symptoms after deposing faeces on the adaxial surface of the leaves may be explained by differences in the physical and physiological structure of abaxial and adaxial leaf surfaces, such as amount of surface waxes, thickness of cuticule, and lower number of stomata on the adaxial surface (Daniel and Boher, 1981b; Bani, 1990b; Daniel, 1991; Zinsou, 2001). Wounds are caused by human activities as well as by insects, wind, hail, extremes of temperature and light. The external barriers of the host may also be broken temporarily as a natural consequence of plant growth and development. Also Shelby et al., (1999) found that direct inoculation of cucurbits with *Erwinia tracheiphila* (Watterson) in caged bioassays was influenced by factors such as size of wounds and wounding before inoculation. Brust (1997) reported that expositing plants to beetles for 72 h, as opposed to shorter intervals, increased the frequency of transmission of *E. tracheiphila*. In cassava fields, *Z. variegatus* faeces are commonly found on the adaxial surfaces of leaves or on the soil, where the faeces are moistened by rain or dew. Under such conditions, multiplication of *X. campestris* pv. *manihotis* may be initiated and constitute the secondary inoculum. Strong winds or wind-driven rains may transport *X. campestris* pv. *manihotis*-infested faeces and contaminated insects (e.g. younger larval instars) within and among plantings of susceptible crops, and thus facilitate the spread of bacteria and the establishment of new infection foci. More precisely, rain droplets could run down from the adaxial surface and reach the under-surface of the same leaf containing more stomata for bacterial entrance (Zinsou, 2001), and therefore contribute to the development of symptoms. The effect of insect transmission on long-distance spread of Xanthomonad pathogens of cassava and cowpea was not investigated. We suppose that the number of viable *X. campestris* pv. *manihotis* retained on the mandibles of *Z. variegatus* fed on infected cassava plants was in general too low to be transmitted to healthy plants. *Schistocerca nitens* (Thunberg) (Orthoptera: Acididae) mouthparts retained

relatively small numbers of *Clavibacter x. cynodontis* (Davis, Gillaspie) even when large numbers were applied (Barbeheim and Purcell, 1993). Nevertheless a survival of *X. campestris* pv. *manihotis* of up to 7 days in/on *Z. variegatus* and five weeks in faeces of *Z. variegatus* (see under 5.3) would allow a transport at least to adjacent fields. We also demonstrated that under field conditions, the transmission was relatively high as well as the transmission in the greenhouse to wounded and intact leaves. When *Z. variegatus* fed on leaves with high *X. campestris* pv. *manihotis* population, the probability of transmission and colonisation of healthy plants should be considerable.

Contrary to our transmission results, Shelby et al., (1999) using ELISA assays, showed that, although *Acalymma vittatum* Fab. (Col.: Chrisomelidae) harboured *E. tracheiphila* (Smith) in sufficient numbers, a transmission of the disease in single-beetle bioassays was not achieved.

Cowpea bacterial blight

We could not find bacterial blight symptoms on cowpea plants after transfer of contaminated *Z. variegatus* under greenhouse conditions. However, an epiphytic *Xanthomonas campestris pv. vignicola* population of up to 8.9×10^4 cfu/g leaf was transferred by contaminated *Z. variegatus* from infected to healthy leaves. Also Barbeheim and Purcell, (1993) demonstrated that none of the leaf-chewing insects (*Melanoplus sanguinipes* Fab. [Orth.: Acrididae], *Diabrotica undecimpuncta* Mannerheim [Col.: Chrysomelidae] *Mythimna unipuncta* Haworth [Lep.: Noctuidae]) or xylem-sucking insects (*Carneocephala fulgida* Nottingham and *Draeculacephala minerva* Minerva Ball [both Hem.: Cicadellidae]) transmitted *C. x. cynodontis* when transferred in groups from maize to un-colonised to uncolonised plants. Moreover, Wayadande and Fletcher (1995) suggested, that the loss of transmissibility by spiroplasma might be due to the inability of the mollicutes to utilise hemolymph nutrients or to multiply in the haemocoel. Although the transmission of *X.*

campestris pv. *vignicola* by *Z. variegatus* could not be demonstrated by symptom development on formerly healthy leaves under greenhouse conditions in the dry season, when relative humidity in the greenhouse is rather low, the population of the pathogen which survived as epiphytes on the leave surfaces may constitute the inoculum necessary for the development of the disease when conditions become more favourable, e.g. at the onset of the rainy season. Thus, environmental conditions after the inoculation have a strong impact on the subsequent disease development (Purcell, 1982). Epiphytic growth of bacterial pathogens generally is favoured by high humidity and warm temperatures (Leben, 1963 &1974). If we consider that, as for many pathogenic diseases, plant infection being a dose-dependent process (Lukezic et al., 1997), then even low levels of inoculum e.g. on epiphytic population of pathogenic bacteria may contribute to transmission under favourable conditions. The probability of infection may increase by incremental addition with increasing insect density. We did not check the transmission of *X. campestris* pv. *vignicola* by faeces. However, the fact that we revealed epiphytic population on leaves, and the determination of the infectivity titer at 10^4 cfu/ml, which should correspond to a bacterial population of less than 10^4 cfu/g leaf, suggest that under favourable conditions symptoms may appear also under low inoculum pressure. A frequency of 8% was observed when approximately 10^4 cfu of *E. tracheiphila* (Smith) were present on the mandibles of *S. nitens* (feeding on stems of cucumber (Barbeheim and Purcell, 1993). In the field, bacterial blight symptoms are prevalent in form of leaf spots, blight and canker which all may additionally carry bacterial exudates. In a cowpea field, many insects were contaminated with *X. campestris* pv. *vignicola* (see under 3.3.2). Once bacterial ooze is extruded, many different insect species are attracted by the exudates (Steiner, 2000) and the dispersal of inoculum from plant to plant begins, leaving colonies of bacteria, wherever insects walk.

The transmission is also influenced by feeding habits, since uninterrupted feeding may further reduce the probability of transmission as a result of the ingestion and dilution of the inoculum on the mouthparts (Barbehenn and Purcell 1993). Studying insects transmission of *Ralstonia solanacearum* (Smith) to banana, Buddenhagen and Elsasser (1962) suggested that success of insects as vectors of bacterial plant pathogens depends upon (i) insect number and activity, (ii) abundance, viability, and virulence of bacterial inoculum, and (iii) the frequency and susceptibility of infection sites. Thus, our results on the transfer and build-up of an epiphytic population and low infectivity titer suggest, that under favourable conditions the transmission of *X. campestris* pv. *vignicola* by insects may play a role in bacterial blight epidemiology.

5 Survival of *X. campestris* pv. *manihotis* in/on *Z. variegatus*

5.1 Introduction

In West Africa, small-scale farmers grow cassava and cowpea as staples for carbohydrate and protein supply, respectively. During the rainy season, high humidity and warm temperature favour epidemiology of bacterial blight of cassava and cowpea caused by *X. campestris* pv. *manihotis* (Bondar, 1912) and *X. campestris* pv. *vignicola* (Burkholder, 1944) (Dye 1978) respectively, two important diseases in cassava and cowpea growing areas worldwide. During this period, insect feed and injure the plants`foliage and stems. Insect dissemination of cassava and cowpea bacterial blight was suggested by various authors (Hayward and Waterston, 1965 cited by Walter and Vakili, 1977; Bani, 1990b). In some case, insect vectors may not only be involved in transmission, but also provide substrates for the growth of bacteria, which in turn adversely affect the host plant as demonstrated for a saprophytic species of *Xanthomonas*, growing on substrate provided by fruit fly growing on substrate provided by fruit fly larvae in coffee berries (Stolp 1960) cited by Damon, (2000).

The multiplication of plant pathogenic mollicutes injected into non-vector insects has been demonstrated for aster yellows (Sinha and Chiykowski, 1967), X-disease (Purcell 1981), *Spiroplasma citri* Lines (Whitcomb et al., 1973), and corn stunt (Williamson and Whitcomb, 1975). Plant pathogenic prokaryotes may provoke a range of interactions with their vectors, ranging from beneficial to harmful (Maramorosch and Jensen, 1963; Chiykowski, 1981). Some mollicute plant pathogens are harmful to their leafhopper vectors (Purcell, 1982). In contrast, the aster yellows agent seems not to directly affect vector longevity or fecundity (Severin, 1945). The aster leafhopper, *Macrosteles quadrilineatus* (Forbes) (Hem.: Cicadellidae), preferred to feed on and survived longer on aster yellows-infected celery than on healthy celery (Peterson, 1973 cited by Purcell, 1982). The causal agent of potato blackleg,

E. carotovora (Jones, Winslow et al.) was reported to multiply and survive the winter in the alimentary canal of the seed corn maggot, *Delia platura* (Meigen) (Dipt.: Anthomyiidae) (Leach, 1940; Carter, 1973).

Some studies showed that *X. campestris* pv. *manihotis* (Bondar, 1912) can survive in the intestinal canal of *Zonocerus variegatus* (L.) (Orth.: Acrididae) (Daniel et al., 1980; Bani, 1990b). However, no details about the survival in/on the insects over time remain still unknown, and only few studies on the interaction of the disease with the insect are available. Therefore, the main objective of our studies was to determine the survival of *X. campestris* pv. *manihotis* in/on *Z. variegatus* in the faeces, and on the insect organs. Additionally, the effect of feeding on infected versus healthy leaves on the life stages of *Z. variegatus* was investigated.

5.2 Materials and methods

5.2.1 Survival of *X. campestris* pv. *manihotis* in/on *Z. variegatus*

After feeding on *X. campestris* pv. *manihotis*-infected leaves for one week in the greenhouse, insects were transferred onto healthy cassava plants. Fifteen insects were collected each week for two weeks for detection of the pathogen on the mandibles, the legs, in the alimentary canal, the peritrophic membrane and in the faeces. Seven insects were combined in one sample, and isolation and isolation and identification of bacteria was carried out as described under 3.2.4.

5.2.2 Survival of *X. campestris* pv. *manihotis* in naturally contaminated faeces

Faeces were collected from insects that had previously fed on bacterial blight-infected cassava leaves in the greenhouse for one week. One part of the faeces was exposed to sunlight while the remaining part was kept under laboratory condition (25°C). One mg of the faeces was

collected once per week over six weeks and the number of bacteria was determined following the method described under 3.2.4.

5.2.3 Survival of *X. campestris* pv. *manihotis* in artificially contaminated faeces

Non-contaminated faeces from insects fed on *V. amygdalina* were collected from insect rearing cages. Faeces were sprayed with asuspension (10^8 cfu/ml) of the antibiotics-marked strain *X. campestris* pv. *manihotis* GSPB 2711. The infested faeces were kept in glass Petri dishes under greenhouse conditions (25-30°C and 65-95% RH). Every second day over five days, 1 mg of the faeces were collected for detection of *X. campestris* pv. *manihotis* following the protocol described under 3.2.4.

5.2.4 Survival of *X. campestris* pv. *manihotis* through *Z. variegatus* passage

Thirty *Z. variegatus* were fed on cassava leaves showing water-soaking symptoms (not necrotised) in the greenhouse. Ten hours after feeding of insects on infected leaves, faeces were collected. Weight of the leaves before and after feeding (i.e. the leaf residues) was recorded. In infected 'control' leaves showing a similar extent of blight symptoms, the number of bacteria per g leaf was determined. In parallel, the number of bacteria in faeces was quantified using the method described under 3.2.4. To determine the number of bacteria surviving the passage through *Z. variegatus*, the number of bacteria in fed leaves per insect was calculated using the number counted in aliquots of the similarly infected 'control ' leaves and compared to the number counted in the collected faeces per insect. Faeces were collected over a period of ten hours after feeding.

5.2.5 Influence of X. campestris pv. manihotis on Z. variegatus

Different larval instars of *Z. variegatus* were collected in farmers' fields near Cotonou, Benin. For 24 h the collected insects were fed on *V. amygdalina* leaves to reduce the number of micro-organisms in the intestines of the insects. *V. amygdalina* is reported to have an

antibiotic effect (Mintesnot and Mogessie, 1999; Gebreyohannes and Drassar, 1992). Then, insects of larvae stages L2 (6 insects) L3 (29 insects) L4 (15 insects) were fed in individual Petri dishes with either healthy or infected cassava leaves under greenhouse conditions over a period of three months, i.e. until they reached the seventh larval instar. The time of development from L_2 to L_7, L_3 to L_7 and L_4 to L_7, the weight of the fed leaves and the mortality during the experiment were recorded and compared between the two treatments.

5.2.6 Diet sampling

Plant fragments found in the faeces were determined using the micro-histological method developed by Le Gall and Gillon (1989). Grasshoppers were collected in the morning after 9 a.m., in cowpea field in the center and the south department. Their faeces were placed in individual plastic tubes. The collected faeces were dried at 50°C. For microscopical observations, faeces were re-hydrated, treated with hypochlorite then dried in alcohol (from 70 to 100%) and finally mounted on microscope slides with Euparal. Slides were observed at a magnification of 40-100 times, enabling us to detect residues of plants, which were then identified by comparing the test slides to control slides with cowpea plant tissue.

5.3 Results

5.3.1 Survival of *X. campestris* pv. *manihotis* in/on *Z. variegatus*

Insects that had previously fed on *X. campestris* pv. *manihotis*-contaminated leaves were transferred onto healthy cassava plants under greenhouse conditions to study the survival time of the bacteria on and in their organs. On the transfer day, all the organs and the faeces carried considerable numbers of the pathogen (Table 5.1). After one week, no more bacteria were detected on mandibles, legs and on the peritrophic membrane. However, some bacteria survived in the faeces, and few were also found in the alimentary canal. After two weeks no more bacteria were detected.

Table 5.1: Survival of *X. campestris* pv. *manihotis* in/on *Z. variegatus* after feeding the insects with infected cassava leaves only and subsequent transfer to healthy cassava plants

		Day of transfer	1 week after transfer		2 weeks after transfer	
Organs	Replication[a]	Cfu/insects[b]	Mean	Cfu/insects	Mean	Cfu/insects	Mean
Mandibles	A	990 ± 10	940	0	0	0	0
	B	890 ± 12		0		0	
Legs	A	1,110 ± 10	1,055	0	0	0	0
	B	1,000 ± 8		0		0	
Intestine	A	1,145 ± 5	1,095	5	3	0	0
	B	1,045 ± 12		0		0	
Peritrophic membrane	A	1,915 ± 8	1,280	0	0	0	0
	B	645 ± 5		0		0	
Faeces	A	++++[c]	++++	+	+	0	0
	B	++++				0	

[a] One replication consisted of seven insects which were pooled in one sample for detection
[b] Cfu = Colony forming units; data presented as mean ± SE
[c] ++++= uncountable high number of Cfu, += uncountable number of Cfu

5.3.2 Survival of *X. campestris* pv. *manihotis* in naturally contaminated faeces of *Z. variegatus*

X. campestris pv. *manihotis*-contaminated faeces of *Z. variegatus,* collected from bacterial-blight inoculated (plot A, see under 2.5), were kept in Petri dishes under controlled conditions to determine the survival time of the bacteria. The number of *X. campestris* pv. *manihotis* in the faeces decreased progressively with time, and after five weeks no more bacteria were detected (Table 5.2). However, when contaminated faeces were exposed to sunlight, bacterial numbers decreased rapidly and reached zero before two weeks.

Table 5.2: Survival of *X. campestris* pv. *manihotis* in naturally infested faeces of *Z. variegatus* under controlled conditions and under exposure to sunlight

	Cfu/mg in faeces [a]	
Date [b]	Controlled conditions[c]	Exposure to sunlight [d]
Day 1	Ne[e]	$7.1 \times 10^7 \pm 1.4 \times 10^7$
Week 1	Ne	$6.3 \times 10^4 \pm 1 \times 10^4$
Week 2	$2.6 \times 10^5 \pm 1.0 \times 10^5$	0
Week 3	$8.8 \times 10^4 \pm 2.4 \times 10^4$	Ne
Week 5	$2.1 \times 10^2 \pm 0.3 \times 10^2$	Ne
Week 6	0	Ne

[a] *X. campestris* pv. *manihotis* populations at each sampling week: cfu are means of colonies on two plates ± SE

[b] Time after keeping infested faeces under laboratory conditions and outside

[c] Faeces from *Z. variegatus* fed on infected cassava plants kept at 25 °C in laboratory

[d] Faeces from *Z. variegatus* fed on infected cassava plants kept in open air outside, nd.= not done

[e] Ne = not examined

5.3.3 Survival of *X. campestris* pv. *manihotis* in artificially contaminated faeces of *Z. variegatus*

Z. variegatus faeces were sprayed with the antibiotics-resistance marked strain *X. campestris* pv. *manihotis* GSPB 2511. Twenty-four hours after the inoculation of the faeces, 2.2×10^5 cfu/mg faeces were detected. The number decreased to 1.5×10^4 and zero after 48 and 96 h, respectively (Table 5.3).

Table 5.3: Survival of *X. campestris* pv. *manihotis* in artificially infested faeces under controlled conditions

Date [a]	Replication [b]	Cfu/mg [c]	Mean
	A	$2.2 \times 10^5 \pm 1.0 \times 10^5$	
1st dpi	B	$2.4 \times 10^5 \pm 0.8 \times 10^5$	2.2×10^5
	C	$2.2 \times 10^5 \pm 1.1 \times 10^5$	
	A	$1.6 \times 10^4 \pm 0.5 \times 10^4$	
3rd dpi	B	$2.0 \times 10^4 \pm 0.5 \times 10^4$	1.5×10^4
	C	$1.0 \times 10^4 \pm 1.0 \times 10^5$	
	A	0	
5th dpi	B	0	0
	C	0	

[a] Days after artificial infestation of faeces with bacterial suspension of strain GSPB 2511 at concentration of 10^8 cells/ml.
[b] Each replication consisted of 1 mg of faeces.
[c] Cfu = colony forming units, data presented as mean ± SE.

5.3.4 Survival of *X. campestris* pv. *manihotis* through insect passage

After feeding the insects on bacterial blight-infected leaves, the number of bacteria surviving in the faeces was by a factor of 10^1 to10^6 cfu lower compared to the number of bacteria in the leaves, which had been fed to the insects (data not shown. See also Annex 1). Significantly higher numbers of bacteria were recorded in the leaves than in the faeces (Table 5.4).

Table 5.4: Number and percentage of *X. campestris* pv. *manihotis* detected in faeces of *Z. variegatus* after feeding with infected leaves compared to the number in fed leaves

Leaves	Faeces	% loss
1.3×10^9a	5.83×10^7b	95
T= 24.75	$P < .0001$	N = 22

Means followed by different letters are significantly different at 5%
Total number of *Xanthomonas campestris* pv. *manihotis* (mean of 22 repetitions)

5.3.5 Influence of *X. campestris* pv. *manihotis* on *Z. variegatus*

Second, third and fourth larval instars of *Z. variegatus* were fed with either *X. campestris* pv. *manihotis*-infected or healthy leaves for a period of three months, enabling them to reach the seventh larval instar. Only in the experiment which started with the third larval instar of *Z. variegatus*, a significantly higher consumption of healthy compared to *X. campestris* pv. manihotis-infected leaves was recorded (Table 5.5). Moreover, in the two experiments which started with using second and third larval instars of *Z. variegatus*, a significantly longer development time until reaching the seventh larval instar was recorded when grasshoppers were fed with *Xcm*-infected cassava leaves (Table 5.6).

Table 5.5: Plant biomass consumption (in g) of healthy and *X. campestris* pv. *manihotis* - infected cassava leaves over a period of three months by different larval development stages of *Z. variegatus*

Treatment	Larval instars of *Z. variegatus*		
	L2-L7	L3-L7	L4-L7
Healthy	8.6 ± 0.6a[a]	8.8 ± 0.3a	7.8 ± 0.4a
Infected	9.6 ± 0.4a	8.1 ± 0.1b	6.7 ± 0.5a

[a] Means (± SE) per column followed by the same letter are not significantly different (Turkey, $P < 0.05$).

Table 5.6: Development time of different larval development stages of *Z. variegatus* when fed with healthy and *X. campestris* pv. *manihotis*-infected cassava leaves over a period of three months

	Larval instars of *Z. variegatus*					
	L2-L7[a]		L3-L7		L4-L7	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
Mean[b]	47.3 ± 14.6	101 ± 2.1	65.4 ± 1.9	180.1 ± 2.3	52.1 ± 2.3	55.9 ± 2.3
P-values[c]	0.0016		0.001		0.2744	

[a] Number of different *Z. variegatus* instars L2 = 6, L3 = 29, and L4 = 15

[b] Mean ± SE.

5.3.6 Dietary study

The insects shown a specific In dietary studies of thirty grasshopper species collected in cowpea fields, cowpea residues were detected in the intestines of 14 species (Table 5.7) and in one individue of *Cantoncylus* spp., *Helipodinae spp.*, *Metichirista* spp., *Spathos spp.*, *Truxalis grandis*, Klug indicating that these grasshopper species can potentially transfer *X. campestris* pv. *vignicola*, when feeding on bacterial blight-infected cowpea leaves.

Table 5.7: Residues of cowpea in grasshoppers of different species collected in a cowpea field

Numbers	Species	No. of insects with residues of	
		Cowpea	Others
8	*Acriada bicolour* Thunberg	4	4
6	*Aiolopus thalassinus* Fab.	1	5
12	*Cantatops stramineus* Walker	3	8
7	*Chrotogonus senegalensus* Kr.	5	2
30	*Coryphosinae stenoptera*	12	18
12	*Eyprocnemis plorans*	5	7
4	*Gymnogotrus* spp.	3	1
8	*Mumbe* spp.	2	6
4	*Oedaleus* spp. Fieber	3	1
15	*Oxya hila hila* Serville	4	11
20	*Pyrgomorpha cognata* Kr.	12	8
11	*Pyrgomorpha vignaudie Guer.*	5	6
21	*Trilophida contubatus* Walk.	5	16
198	*Zonocerus variegatus* L.	103	95
26	Non-identified (larvae)	5	21

5.4 Discussion

Unlike xylem-limited bacteria (e.g., *Erwinia tracheiphila* Watterson) that can survive for extended periods in insect vectors (Harrison et al., 1980; Purcell, 1989), our study on *Z. variegatus* revealed a limited survival time on mandibles and legs of less than one week, and of more than five weeks in the digestive system and in the faeces. Moreover, we did not record any multiplication of *X. campestris* pv. *manihotis* in/on *Z. variegatus*. The observed quick reduction in bacterial numbers may have been due to desiccation and the presence of enzymes or other microflora in the alimentary canal which inhibited the survival of *X. campestris* pv. *manihotis*. Bani (1990b) detected *X. campestris* pv. *manihotis* in the

alimentary canal two months after feeding *Z. variegatus* on infected plants using indirect immunofluorescence, and Daniel and Boher (1985b) suggested that *X. campestris* pv. *manihotis* could survive and multiply in the alimentary canal of *Z. variegatus* during the dry season, when no cassava bacterial blight lesions were observed on the leaves. Garcia-Salazar et al. (2000b) observed that in *E. tracheiphila* (Smith) infections in *Acalymma vittatum* F. (Col.: Chrysomelidae) the bacteria preferentially colonised the peritrophic membrane of the alimentary canal of the beetles. Similarly, Purcell and Mclean (1979) observed a multiplication of *Xylella fastidiosa* Almond Dixon, the bacterial agent of Pierce's disease, in the intestinal canal of *Graphocephala atropunctata* (Signoret) (Hom.:_Cicadellidae), and Petri, (1909 & 1910, cited by Carter, 1973) reported that the oesophagus and the intestinal canal of larvae of *Dacus oleae* Gmelin (Dip.: Tephritidae) harboured *Pseudomonas savastonoi* (Smith), the causal agent of olive knot disease. In our studies, the survival time of *X. campestris* pv. *manihotis* in faeces depended on the way of infestation of the faeces, i.e. naturally by feeding or artificially by adding a bacterial suspension, and on the conservation conditions. *X. campestris* pv. *manihotis* survived more than five weeks in naturally contaminated faeces under controlled conditions (25°C), but only one week in naturally contaminated faeces and four days in artificially inoculated faeces, when exposed to sunlight in the field. A negative effect of ultraviolet (UV) radiation on the survival of pathogenic bacteria on leaf surfaces has been reported by Leben (1963) and Schuster and Coyne (1975). The relatively long survival time of *X. campestris* pv. *manihotis* in naturally contaminated *Z. variegatus* faeces may be explained by the fact that the bacteria were still in clumps, and were protected by a membrane. In contrast, when spraying, only single cells of the bacteria were present in the suspension, and thus the bacteria could have easily died because of external factors such as UV radiation. According to Daniel and Boher (1985), *X. campestris* pv. *manihotis* can survive for two months in contaminated faeces under laboratory conditions

(25°C, 70% RH). Also, Walter and Vakili (1977) reported that the inoculum on the exoskeleton of naturally infected insects, inoculum present in their faeces, and/or inoculum in regurgitated fluids, is reduced quickly to low levels through desiccation and exposure to UV radiation.

The bacteria survived the passage through the insects in sufficient numbers, i.e. between 10^3 and 10^8 Cfu/g faeces for infection of cassava plants under favourable conditions. Nothing is known about the potential pathogenicity of *X. campestris* pv. *manihotis* and *X. campestris* pv. *vignicola* to *Z. variegatus*. According to Purcell (1982), plant pathogenic prokaryotes may provoke a range of interactions with their vectors, ranging from beneficial to harmful when they enter in the insect (Wayadande and Fletcher, 1995). However, only a small percentage of prokaryotes that are associated with insects appear to be harmful to them (Purcell, 1982). Our study showed that *Z. variegatus* readily fed on infected as well as on healthy cassava leaves. However, in the experiment which was started with L3 instars of *Z. variegatus*, grasshoppers fed significantly less infected than healthy cassava leaves. Yet, in the other trials in this series of experiments, no such results were obtained. Hence, from this data we can not clearly conclude a specific avoidance behaviour in *Z. variegatus* of blight-infected compared to healthy cassava leaves. Bani (1990b) reported that 61.9% of the first, second, third and fourth larval instars of *Z. variegatus* fed on *X. campestris* pv. *manihotis*-infected leaves, hence indicating a certain preference of *Z. variegatus* for blight-infected cassava leaves. Similarlily the aster leafhopper, *Macrosteles fascifrons* Stal. (Hom.: Cicadellidae) prefers to feed on and survives longer on aster yellows-infected celery than on healthy celery (Severin, 1934). In our study the total development was significantly prolonged when young larval instars of *Z. variegatus* had fed on *X. campestris* pv. *manihotis*-infected cassava leaves. Possibly infected leaves may contain substances that interact with the development of young grasshopper larvae. In contrast, Kunkel (1936) and Severin (1946) studing the longevity and certain other

life history parameters of leafhoppers reported that feeding on virus-infected aster plants did not affect the longevity and/or fecundity of the vectors. However, more information on the potential effects of *X. campestris* pv. *manihotis* on its vector *Z. variegatus* are needed for a thorough understanding of the precise nature of this pathogen-vector relationship.

The presence of plant species in the diet is best defined by vegetal remains in the gut or the faeces (Brusven and Mulkern, 1960; Hummelen and Grillon, 1968). Our findings suggest that the tested grasshopper species can potentially transfer *X. campestris* pv. *vignicola* when feeding on bacterial blight-infected cowpea leaves. However, in future studies the potential impact of insect vectors for the epidemiology of *X. campestris* pv. *manihotis* and *X. campestris* pv. *vignicola* needs to be investigated.

6 General discussion and conclusions

The major goal of this study was to demonstrate the transmission of the bacterial pathogens of *Xanthomonas campestris* pv. *manihotis* (Bondar, 1912) and *Xanthomonas campestris* pv. *vignicola* (Burkholder, 1944) of cassava and cowpea, respectively by insects in general, and by *Zonocerus variegatus* in particular, under field and greenhouse conditions in West Africa. We sought to expand our understanding of the location, of the two plant pathogens within their vectors and the mechanism of transmission. Our studies indicated that *Z. variegatus* and other insect species were contaminated during their movement in the infected fields leaves. *X. campestris* pv. *manihotis* and *X. campestris* pv. *vignicola* were isolated from the mandibles, legs, and the alimentary canal up to 1,280 Cfu/insect, and in the faeces up to $2.2x10^5$ Cfu/ml of *Z. variegatus*. Daniel et al., (1980), Daniel and Boher (1985) and Bani, (1990b) made similar observations, though they did not demonstrate the transmission of the bacteria under field conditions.

The immunofluorescence technique using monospecific antisera provided clear images of bacterial cells attached to the fore-and hindguts of *Z. variegatus*. Schaad and Kendrick (1975) recommended **indirect** immunofluorescence as a reliable method which they used to confirm the identification of *X. campestris* isolated from crucifer seeds. Thus, the antisera used were sensitive enough to detect also small number of *X. campestris* pv. *manihotis* in tissues of a single grasshopper, allowing detection at levels that escaped detection using agar medium. The development of cassava bacterial blight symptoms in the greenhouse after deposing infested faeces on cassava leaves demonstrated the transmission of *X. campestris* pv. *manihotis* by *Z. variegatus*. The percentage of transmission was higher when the plants were damaged (90%) compared to the non-damaged plants (46.7%). In constrast, Bani (1990b) did not obtain symptoms when *X. campestris* pv. *manihotis* contaminated-faeces were deposit on intact cassava leaves.

Once in the field, *Z. variegatus* defecates on the adaxial surface of the leaves or on the plants debris on the soil. When the environmental conditions are favourable, the bacteria can be dispersed by rain either by direct splashing or as aerosols carried on even modest winds. Moreover, we found that many insects and honey bee was contaminated with *X. campestris* pv. *vignicola* during their movement in a cowpea field. Therefore, as honeybees arrive to collect pollen, the bacteria are picked up on their body hairs and are then subsequently moved to other flowers in the field or in the adjacent field.

Our study showed that *X. campestris* pv. *manihotis* survived less than one week on the mandible and legs, up to one week in the digestive system and up to five weeks in the faeces. We also found that the numbers of bacteria surviving in the faeces through the insects passage was significantly reduced. This reduction may be due to the influence of external conditions such as UV radiation and internal conditions such as enzymes present in the alimentary canal. A similar reduction of other plant-pathogenic bacteria was reported by other authors (e.g. Leben, 1963 & 1974; Schuster and Coyne, 1975).

Additional trials showed that *X. campestris* pv. *manihotis*-infested leaves affect negatively the total development time of second and third instar larvae of *Z. variegatus*. The development time increases when feeding on infected leaves. Also the third instars showed a significant reduction after infected leaf consumption. However, for the second and fourth instars of the insects we did not obtain such results. Therefore, a further investigation is needed on the influence of *X. campestris* pv. *manihotis* on the insect.

Our results showed that insects, especially *Z. variegatus*, can be important vectors of both cassava and cowpea bacterial blight, and may substantially contribute to the dissemintion of the two diseases. The survival of the bacteria and the probability of tzransmission to healthy cassava plants play an important role in the dissemination by insects. Therefore, our

recommendations to plant breeders are to select cassava varieties resistance to *Z. variegatus*. Further research is needed to develop integrated measures for control of *Z. variegatus*, to be recommended to farmers.

7 Summary

Cassava bacterial blight is an epidemic disease, which has recently gained importance in all cassava growing areas of Africa. The causal agent of the disease is *Xanthomonas campestris* pv. *manihotis*. Recent surveys revealed the prevalence of the disease in Benin and Nigeria with regionally severe outbreaks. Significant yield losses caused by *X. campestris* pv. *manihotis* have been recorded in several ecozones of West Africa (Wydra et al., 1998). Cowpea bacterial blight caused by *Xanthomonas campestris* pv. *vignicola* occurs in many African countries and other regions of the tropics and subtropics. The present studies aimed at quantifying the contamination of insects and their faeces with the blight-causing bacteria, the localisation of the bacteria in/on insects, and at demonstrating the infection of plants through contact with insects.

Cassava bacterial blight

X. campestris pv. *manihotis* was isolated from mandibles, legs, and the alimentary canal, and in high numbers from the faeces of *Zonocerus variegatus* (L.) (Orth.: Acrididae). With immunofluorescence microscopy, the pathogen was localised in the guts of *Z. variegatus*. However, no clear differences were observed between the number of bacteria in fore- and hindguts of *Z. variegatus*. Symptom incidence was higher in plots to which *Z. variegatus* deriving from cassava bacterial blight-infected fields were transferred to compared to control plot. When contaminated faeces were deposited on damaged and non-damaged cassava leaves, typical symptoms appeared after five and seven days, respectively with a percentage of transmission of 90% and 46.7 %, respectively.

The pathogen could survive more than five weeks in faeces of *Z. variegatus* under controlled conditions, but only one week when faeces was exposed to sunlight. Although numbers of bacteria rapidly decreased during the *Z. variegatus* passage, sufficiently high numbers

survived to subsequently cause an infection in cassava, providing weather conditions were favourable for the pathogens.

Cowpea bacterial blight

X. campestris pv. *vignicola* was isolated from three grasshopper species (*Z. variegatus* (L.), *Pyrgomorpha cognata* (Krauss), *Oedaleus spp.* (Fieber)), one Hymenoptera (*Apis mellifera* (L).), and three Coleoptera (*Ootheca mutabilis* (Stahl), *Milabris spp.*(fab.) and *Exochomus troberti* (Mulsant)). Epiphytic *X. campestris* pv. *vignicola* populations of up to 8.9 x 10^4 cfu/ml were detected on leaves after transferring *X. campestris* pv. *vignicola*. An inoculum of 10^4 cfu/ml was demonstrated to be sufficient to induce symptoms. Additionally, the presence of cowpea residues was shown in faeces of different grasshopper species.

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I am solely responsible for the views expressed in this report and hope that the assembled information provides a useful tool for students and scientists working on the same topic in Africa.

Declaration

I, ZANDJANAKOU TACHIN, Martine, hereby declare, that the work presented in this thesis is my own and has not been submitted for a degree in any other University.

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