An evaluation of the endophytic colonies present in pathogenic and non-pathogenic Vanguerieae using electron microscopy

S.L. Stanton [a], J.J.M. Meyer [a,*], C.F. Van der Merwe [b]

[a] Department of Plant Science, University of Pretoria, Pretoria 0002, South Africa
[b] Laboratory for Microscopy and Microanalysis, University of Pretoria, Pretoria 0002, South Africa

A B S T R A C T

Fadogia homblei, *Pavetta harborii*, *Pavetta schumanniana*, *Vangueria pygmaea* (=*Pachystigma pygmaeum*), *Vangueria latifolia* (=*Pachystigma latifolium*) and *Vangueria thamnus* (=*Pachystigma thamnus*) all induce one of the most important cardiotoxicoses of domestic ruminants in southern Africa, causing the sickness gousiekte. All the plants which cause gousiekte have previously been shown to contain bacterial endophytes. However, in this study other plants within the Vanguerieae tribe that have not been reported to cause gousiekte; namely *Vangueria infausta*, *Vangueria macrocalyx* and *Vangueria madagascariensis*, have now been shown to also contain endophytes within the inter-cellular spaces of the leaves. The disease gousiekte is difficult to characterise due to fluctuations in plant toxicity. The majority of reported cases of gousiekte poisoning are at the beginning of the growing season; and thus the plants are thought to be more toxic at this time. By using both transmission and scanning electron microscopy the endophytes within these Vanguerieae plants were compared visually. Using the plant reported most often for gousiekte poisoning, *V. pygmaea*, a basic seasonal comparison of the presence of endophytes was done. It was found that the bacterial endophyte colonies were most abundant during the spring season.

Keywords:
Pavetta
Pachystigma
Vangueria
Gousiekte
Endophyte

1. Introduction

The family Rubiaceae is the fourth largest flowering family with over six hundred genera, of which *Vangueria* is one such genus comprising of over fifty different species (Verstraete et al., 2011). Many of the members of the Rubiaceae family contain endophytes; some endophytes form nodules and others are present within the inter-cellular spaces of the leaves (Van Wyk et al., 1990; Verstraete et al., 2011). Some plant species in the *Vangueria* genus, namely *Vangueria latifolia* Sond. (=*Pachystigma* cf. *latifolium*), *Vangueria pygmaea* (Schltr.) Robyns (=*Pachystigma pygmaeum*) and *Vangueria thamnus* Robyns (=*Pachystigma thamnus*) are known to be pathogenic whereas *Vangueria infausta* Burch. ssp. *infausta*, *Vangueria macrocalyx* (Sond.) Robyns and *Vangueria madagascariensis* J.F. Gmelin (Lantz and Bremer, 2005) are assumed to be non-pathogenic.

Bode et al. (2010) isolated pavettamine from *Pavetta harborii* which is assumed to be the causative toxin responsible for all gousiekte poisoning. Other plants responsible for gousiekte poisoning include *Fadogia homblei*, *P. harborii* and *Pavetta schumanniana* (Fourie et al., 1989; Verstraete et al., 2011). Gousiekte occurs mainly in southern Africa but cases as far as the Democratic Republic of Congo have been reported (Verstraete et al., 2011). The gousiekte disease affects domestic ruminants, mainly cattle and sheep and is a plant induced cardiomyopathy (Botha and Penrith, 2008; Ellis et al., 2010a). After 3–6 weeks of ingestion of one of the plants the ruminant will suddenly die, usually after physical activity. The word 'gousiekte' was translated from Afrikaans literally means 'quick disease' for there is no pre-warning before the ruminant dies from cardiac failure (Fourie et al., 1989; Ellis et al., 2010b). Van Wyk et al. (1990) reported that endophytes were present in *V. latifolia*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus*. One of the important remaining questions is whether the bacterial endophytes are pathogenic and thus inducing gousiekte or if it's just coincidental that endophytes are located in all the gousiekte inducing plants.

Gousiekte was first identified in 1908 but due to the following factors this disease has proved hard to diagnose; varying or lack of symptoms, animal susceptibility differences, loss of toxicity as the plant dries and apparent seasonal toxicity of the plants (Van Wyk et al., 1990; Hay et al., 2008; Bode et al., 2010). Considering that gousiekte inducing plants apparently undergo seasonal toxicity it would be expected that there is a change during the year in either the concentration of toxic compounds present or the number of endophytes which inhabits the inter-cellular spaces of the leaves during the year. We report here on the occurrence of endophytes within the leaves of the toxic and non-toxic *Vangueria* species and the seasonal endophyte variation in the most often reported gousiekte inducing species, *V. pygmaea*.

* Corresponding author. Tel.: +27 12 4202224; fax: +27 12 4206668.
E-mail address: marion.meyer@up.ac.za (J.J.M. Meyer).

2. Materials and methods

2.1. Plant collection

Freshly collected leaf material of *V. infausta*, *V. macrocalyx*, *V. madagascariensis*, *V. pygmaea* and *V. thamnus* was prepared for transmission electron microscopy. All the leaves were collected in the same week of October 2011 in Mpumalanga province. *V. macrocalyx* leaves were collected near Piet Retief (S 27°09′01″, E 30°59′18″). A field outside Lydenburg was the site for both *V. pygmaea* (S 25°12′94″, E 30°19′03″) and *V. thamnus* (S 25°12′92″, 30°19′02″). The rocky hills near Blyde River Canyon were the site

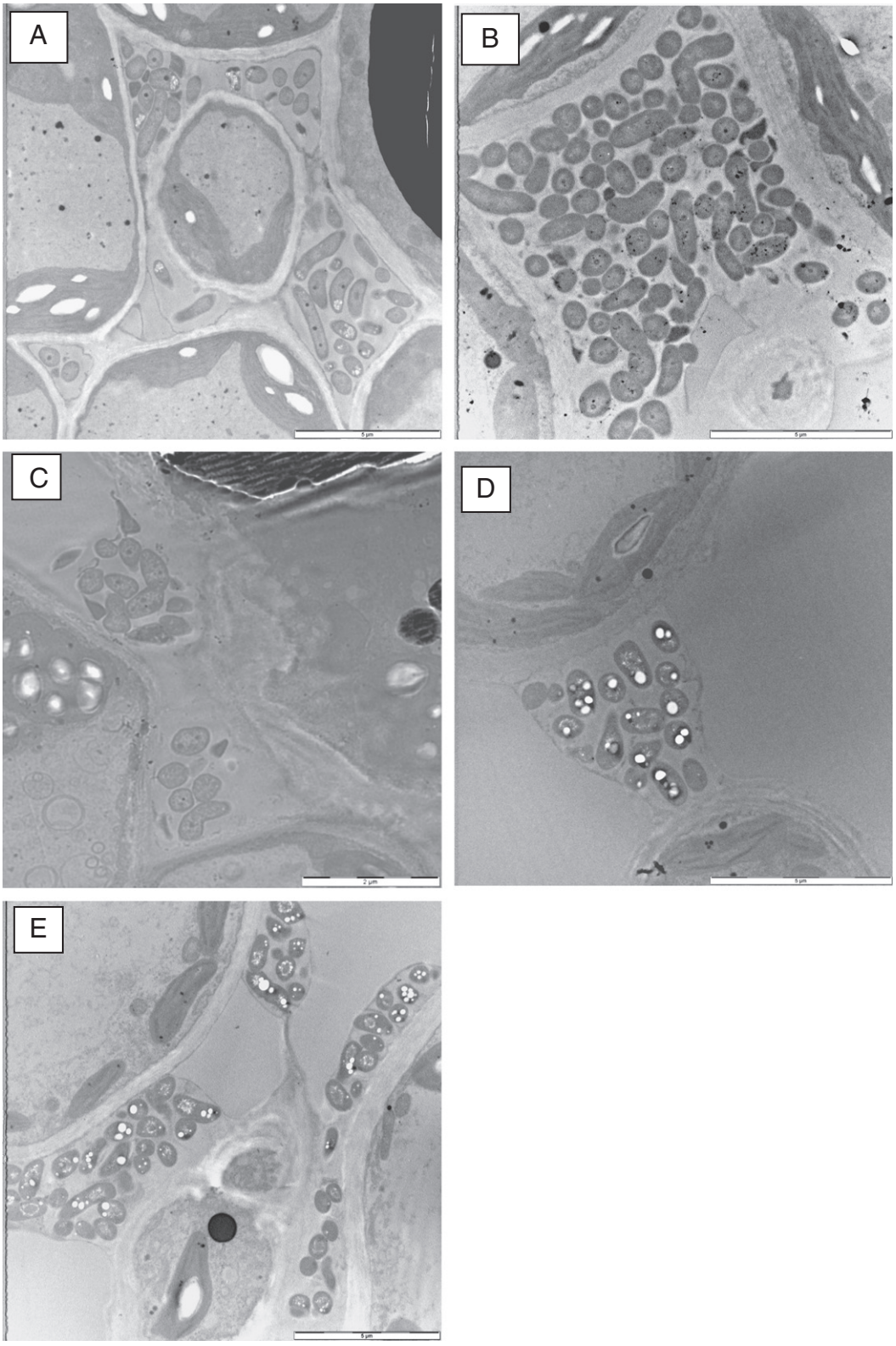

Fig. 1. Endophyte comparison; TEM micrographs of A) *Vangueria infausta*, B) *Vangueria macrocalyx*, C) *Vangueria madagascariensis*, D) *Vangueria pygmaea* and E) *Vangueria thamnus*.

where *V. infausta* (S 24°34′25″, E 30°47′15″) and *V. madagascariensis* (S 24°33′26″, E 30°47′18″) were found. Voucher specimens have been deposited in the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria. The PRU numbers for the plants are: *V. infausta* ssp. *Infausta*, 117607; *V. macrocalyx*, 117601; *V. madagascariensis*, 117611; *V. pygmaea*, 117605; and *V. thamnus*, 117603. Four samples were collected of each plant species, 2× mature fresh leaf and 2× young fresh leaf, and immediately immersed in 2.5% glutaraldehyde in 0.075 M phosphate buffer (Coetzee and Van der Merwe, 1986). These 20 samples were prepared for transmission electron microscopic analysis by following a standard procedure for TEM samples as described below.

2.2. Seasonal plant collection of V. pygmaea

V. pygmaea leaves were collected from a site near the town Rayton in Gauteng (S 25°73′608″, E 028°53′321″), during late October 2010, March 2011, June 2011 and early September 2011. A voucher specimen was placed in the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria and the PRU number is 117989. Six samples were prepared per seasonal comparison for TEM viewing; 3× mature leaves and 3× young leaves. Four samples were also prepared for SEM viewing during each season; 2× mature leaves and 2× young leaves.

2.3. Sample preparation

All five of the *Vangueria* species were prepared as follows. The leaves were cut into 0.5 mm^2 squares. The plant parts were placed into test tubes and fixed in 2.5% glutaraldehyde in 0.075 M phosphate buffer at a pH of 7.4 for between 1 and 2 h at room temperature. Each of the tubes was then rinsed 3 times, 10 min each in 0.075 M phosphate buffer. The samples were fixed in 0.5% aqueous osmium tetroxide in a fume hood and left at room temperature for between 1 and 2 h. The test tubes were rinsed 3 times, 10 min each in distilled water in a fume hood. The samples were dehydrated in ethanol at concentrations of 30%, 50%, 70%, 90% and 3× 100% for 10 min each (Coetzee and Van der Merwe, 1986).

2.4. TEM preparation

The TEM samples were infiltrated with 50% quetol epoxy resin in ethanol for between 30 min and an hour and then infiltrated with 100% pure quetol epoxy resin for 4 h at room temperature. The samples were then polymerised at 60 °C for 39 h and cut into ultrathin sections using a microtome and placed on small copper grids (Coetzee and Van der Merwe, 1986). The TEM used was a JEOL 2100 F.

2.5. SEM preparation

After dehydration the samples underwent critical point drying and were mounted onto the SEM sample stubs. The samples were then coated with gold which makes the sample electrically conductive. After initial viewing of the SEM samples they were split using cello tape to view the interior of the leaves (Coetzee and Van der Merwe, 1986). The SEM used was a JEOL 840.

3. Results

3.1. Endophyte comparison

The five different *Vangueria* species were viewed in order to determine if there were any morphological differences in the endophyte colonies when observed under the transmission electron microscope (Fig. 1). The micrographs of all the *Vangueria* species reveal bacterial endophyte colonisation in the inter-cellular spaces of the leaves. However, the morphology of the colonies appears to be significantly different between the species. As seen in Fig. 1A the bacteria of *V. infausta* are rod shaped, about 1.0–1.5 μm in width and 4.0 μm in length. The morphology of the bacteria present within *V. madagascariensis* (Fig. 1C) appears to be different to that of *V. infausta*; the width is between 0.5 and 1.0 μm and the length 1.0–1.5 μm. This is the first report of endophytes occurring in *V. madagascariensis*. The bacterial endophytes within *V. macrocalyx* are rod shaped, width of 1.0 μm and length of between 2.5 and 3.0 μm as seen in Fig. 1B. The bacterial endophytes within the two gousiekte-causing species, *V. pygmaea* and *V. thamnus* are morpholog-ically quite different from the other three species. The endophytes from the gousiekte-causing species are similar in size and shape, about 0.5 μm wide and 2.0 μm long. Both species' bacterial endo-phytes are embedded in slime-like mucus and contained white polyhydroxybutyrate-like granules (Collins et al., 2012) as shown in Fig. 1D and E.

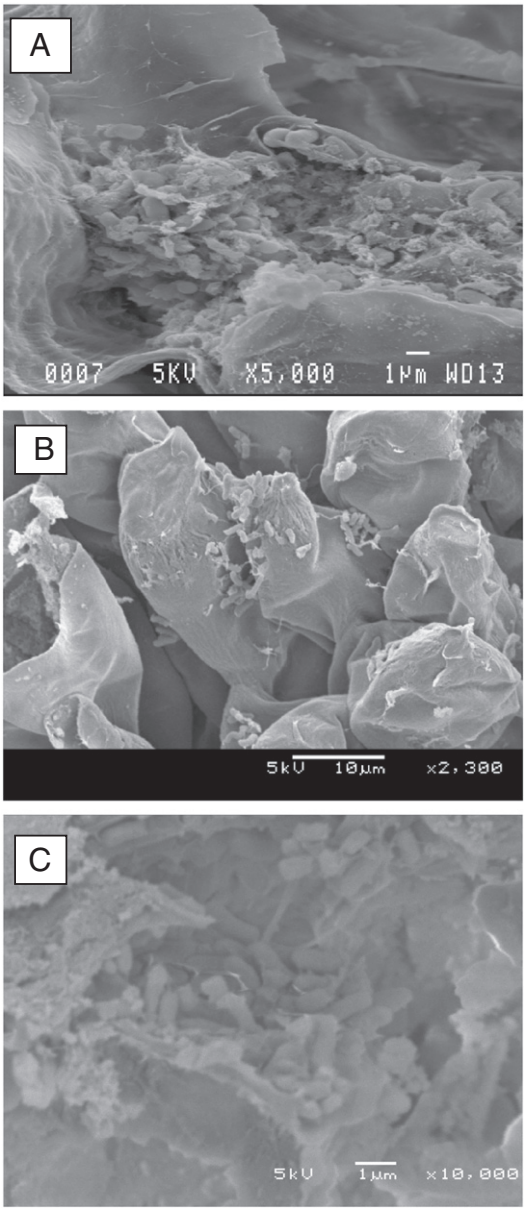

Fig. 2. Seasonal inhabitation of *Vangueria pygmaea* bacterial endophytes as shown by SEM. Micrographs of colonies observed in A) March B) June and C) early September 2011.

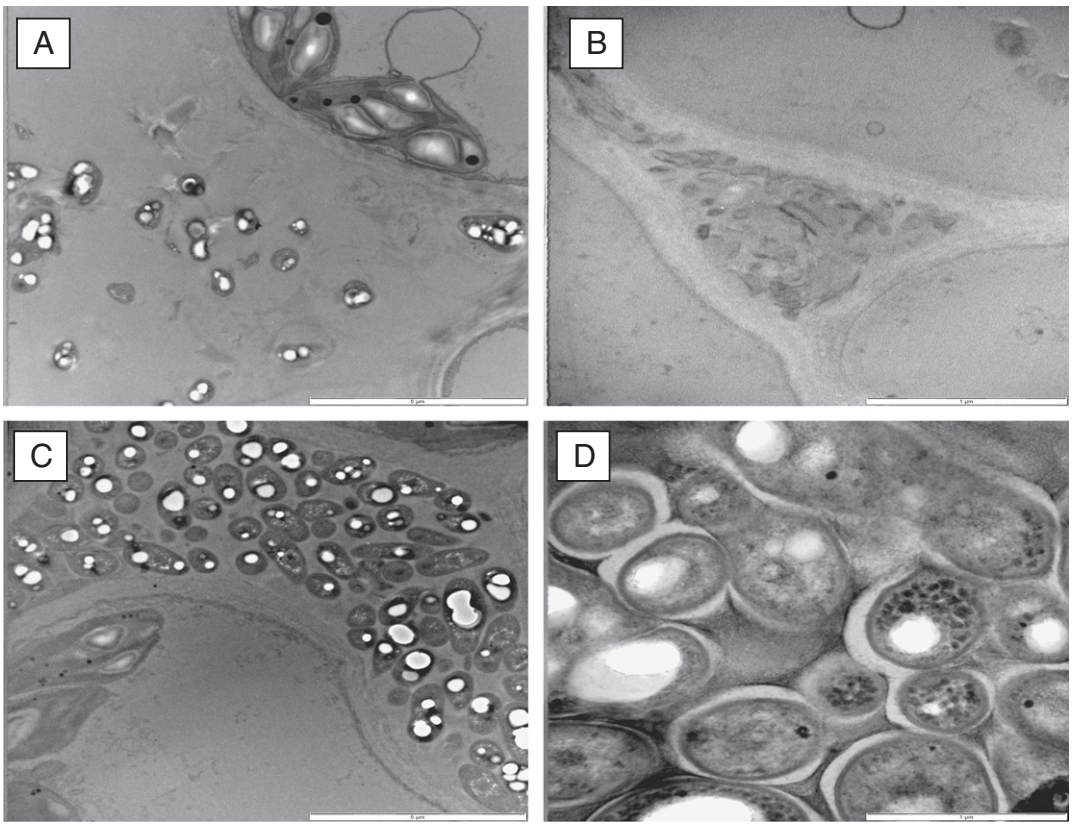

Fig. 3. Seasonal inhabitation of *Vangueria pygmaea* endophytes using TEM. A) March B) June C) early September and D) late October.

3.2. Seasonal endophyte colonisation of V. pygmaea

The SEM micrographs of plants collected during March 2011, June 2011 and early September 2011 are shown in Fig. 2. A change in the number of healthy endophyte colonies could clearly be seen within *V. pygmaea* during different times of the year. Fig. 2A (March) shows a moderate number of bacteria present. Comparing the num-ber of colonies in Fig. 2A with those seen in Fig. 2B (June) it can be noted that there is a significant decrease in the number of bacteria present in the winter. Fig. 2C (September) shows high numbers of bacteria present within the leaves; clearly showing that early September has more endophytic bacteria present than the other months. This was also observed in plants collected in late October.

Fig. 3 shows the TEM micrographs from March 2011, June 2011, early September 2011 and late October 2010. It can be seen that bacterial endophytes are present within the inter-cellular spaces of the leaves of *V. pygmaea* throughout the year. Fig. 3A (March) shows bacterial colonies sporadically spaced during the autumn season whereas very few if any living bacteria can be seen during June, one of the winter months. From these observations it can be said that early September (Fig. 3C) and late October (Fig. 3D) contain the highest numbers of endophytes whereas June (Fig. 3B) contains the least and the endophytes that were present appear to be dead.

4. Discussion

4.1. Endophyte comparison

Bacterial endophyte colonies were observed for the first time in *V. madagascariensis* and are present in the four other *Vangueria* species. Due to the fact that none of these species produce nodules for the bacterial endophytes to reside in; it is not a surprise that the bacterial colonies were observed within the inter-cellular spaces of the leaves. The bacteria are rod shaped and were located throughout the leaves but were more commonly found around the spongy mesophyll parenchyma cells. The bacterial colonies were visually compared with each other and bacteria observed within *V. infausta* were the largest and appeared most similar to those seen within *V. madagascariensis*. It is possible that the bacteria within *V. infausta* and *V. madagascariensis* belong to the same genus. These bacterial colonies did not resemble any of the other endophytes observed within the other three *Vangueria* plants. The bacterial endophytes within the two gousiekte-causing species, *V. pygmaea* and *V. thamnus* resembled each other quite significantly. They contained white possibly polyhydroxybutyrate-like granules, produced a mucus-like substance and they were roughly the same size. The bacteria within *V. macrocalyx* were also observed within the inter-cellular spaces of the leaves. The bacteria appeared different morphologically when compared to the other *Vangueria* species, they were larger and were not embedded in a mucus-like substance as seen in Fig. 1B.

The number of bacterial endophytes generally seen in *V. infausta*, *V. macrocalyx* and *V. madagascariensis* was significantly less than that observed in *V. pygmaea* and *V. thamnus*. All these observations might be significant due to the fact that *V. pygmaea* and *V. thamnus* are the species reported to cause the sickness gousiekte, whereas *V. infausta*, *V. macrocalyx* and *V. madagascariensis* are assumed to be non-toxic. The two species reported to cause gousiekte, *V. pygmaea* and *V. thamnus*, contain endophytes which are very similar; whereas the species which are assumed to be non-toxic, *V. infausta*, *V. macrocalyx* and *V. madagascariensis*, contain endophytes which appear very different to the toxic species.

4.2. Seasonal colonisation

V. pygmaea has been reported to undergo seasonal toxicity fluctuations; being most toxic at the beginning of the growing season

(September) and least toxic during the winter months of June and July (Hay et al., 2008). In order to evaluate if the bacterial endophytes have any correlation in the toxicity of *V. pygmaea* and *V. thamnus* a seasonal comparison of the numbers of bacterial colonies present within the leaves was conducted. In Fig. 3A (March) it can be seen that the condition of the bacteria seems to be 'healthy' and they are present in a moderate number but not tightly packed together. When comparing this observation with that of Fig. 3B, it is clear to see that when bacteria were observed in the winter (June) they appeared to be dying and non-functional. Fig. 3C from early September shows extensive bacterial colonisation within the inter-cellular spaces of the leaves and reveals that the bacteria are encapsulated within a mucilage that appears to be produced by the bacteria. Fig. 3D shows the abundance of bacterial endophytes packed tightly into an inter-cellular space, the bacterial colonies appear to be 'healthy' morphologically during late October (summer).

The results revealed that endophytes are most abundant at the beginning of the growing season, early September, and the least during the winter months in *V. pygmaea*. This correlates with the fact that the plant is reported to be most toxic at the beginning of the growing season, as most poisoning cases are reported during this time and in comparison very little number of cases is reported during the winter. It can be said that these experiments may possibly support the theory that the bacteria and not the plant produces the toxic compound(s) or precursor(s) which make these plants toxic. Further studies need to be conducted to determine if toxic compounds are actually produced by the endophytes.

Another theory which needs to be further evaluated is that gousiekte only affects ruminants and it is possible that the bacterial endophytes produce a precursor compound which only becomes toxic after being metabolised in the rumen. The long period it takes for the ruminant to die could be due to either the toxin having to accumulate in large quantities by addition of more plant material or the bacteria having to multiply in sufficient numbers to produce enough toxin to kill the animal.

In conclusion it can be said that there are still many questions surrounding the plants which induce the sickness gousiekte. It is clear however that other plant species belonging to the Vanguerieae which are reported to be non-toxic, also contain bacterial endophytes within the inter-cellular spaces of the leaves. These might however be of a different species. In order to support the theory that the bacterial endophytes produce the toxin, the same bacterial species or similar compound-producing bacteria need to be isolated from *F. homblei*, *P. harborii*, *P. schumanniana*, *V. latifolia*, *V. pygmaea* and *V. thamnus*.

Acknowledgements

We would like to thank the National Research Foundation of South Africa and Professor T. Coutinho for financially supporting this project.

References

Bode, M.L., Gates, P.J., Gebretnsae, S.Y., Vleggaar, R., 2010. Structure elucidation and stereoselective total synthesis of pavettamine, the causal agent of gousiekte. Tetrahedron 66, 2026–2036.

Botha, C.J., Penrith, M.L., 2008. Poisonous plants of veterinary and human importance in Southern Africa. Journal of Ethnopharmacology 119, 549–558.

Coetzee, J., Van der Merwe, C.F., 1986. The influence of processing protocol on the ultrastructure of bean leaf cells. South African Journal of Botany 52, 95–99.

Collins, A.J., LaBarre, B.A., Wong Won, B.S., Shah, M.V., Heng, S., Choudhury, M.H., Haydar, S.A., Santiago, J., Nyholma, S.V., 2012. Diversity and partitioning of bacterial populations within the accessory nidamental gland of the squid *Euprymna scolopes*. Applied and Environmental Microbiology 78, 4200–4208.

Ellis, C.E., Naicker, D., Basson, K.M., Botha, C.J., Meintjes, R.A., Schultz, R.A., 2010a. A fluorescent investigation of subcellular damage in H9c2 cells caused by pavetamine, a novel polyamine. Toxicology in Vitro 24, 1258–1265.

Ellis, C.E., Naicker, D., Basson, K.M., Botha, C.J., Meintjes, R.A., Schultz, R.A., 2010b. Damage to some contractile and cytoskeleton proteins of the sarcomere in rat neonatal cardiomyocytes after exposure to pavetamine. Toxicon 55, 1071–1079.

Fourie, N., Schultz, R.A., Prozesky, L., Kellerman, T.S., Labuschagne, L., 1989. Clinical pathological changes in gousiekte, a plant-induced cardiotoxicosis of ruminants. The Onderstepoort Journal of Veterinary Research 56, 73–80.

Hay, L., Schultz, R.A., Schutte, P.J., 2008. Cardiotoxic effects of pavetamine extracted from *Pavetta harborii* in the rat. The Onderstepoort Journal of Veterinary Research 75, 249–253.

Lantz, H., Bremer, B., 2005. Phylogeny of the complex Vanguerieae (Rubiaceae) genera *Fadogia*, *Rytigynia*, and *Vangueria* with close relatives and a new circumscription of *Vangueria*. Plant Systematics and Evolution 253, 159–183.

Van Wyk, A.E., Kok, P.D.F., Van Bers, N.L., Van der Merwe, C.F., 1990. Non-pathological bacterial symbiosis in *Pachystigma* and *Fadogia* (Rubiaceae): its evolutionary significance and possible involvement in the aetiology of gousiekte in domestic ruminants. South African Journal of Botany 86, 93–96.

Verstraete, B., Van Elst, D., Steyn, H., Van Wyk, A.E., Lemaire, B., Smets, E., Dessein, S., 2011. Endophytic bacteria in toxic South African plants: identification, phylogeny and possible involvement in gousiekte. PLoS One 6, 1–7.