Evaluation of the efficacy of *Steinernema carpocapsae* against the Red Palm Weevil, *Rhynchophorus ferrugineus* in *Phoenix canariensis*.

Llácer, E.[1], M.M. Martínez de Altube[2] and J.A. Jacas[3]

[1] Unitat Associada d'Entomologia Agrícola UJI-IVIA; Institut Valencià d'Investigacions Agràries (IVIA); Centre de Protecció Vegetal i Biotecnologia; Ctra. Montcada-Nàquera km 4.5; E-46113-Montcada (Spain)

[2] Idebio S.L., Poligono Industrial el Montalvo, Calle Bell 3, E-37008 Salamanca (Spain)

[3] Unitat Associada d'Entomologia Agrícola UJI-IVIA; Universitat Jaume I; Departament de Ciències Agràries i del Medi Natural; Campus del Riu Sec; Av. Vicent Sos Baynat s/n; E-12071-Castelló de la Plana (Spain)

Evaluation of the efficacy of *Steinernema carpocapsae* against the Red Palm Weevil, *Rhynchophorus ferrugineus* in *Phoenix canariensis*.

Abstract

The red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera, Curculionidae), is an important pest of palms. It has recently colonized the Mediterranean Basin where it is a serious problem on ornamental *Phoenix canariensis* (Chabaud) palms. The efficacy of *Steinernema carpocapsae* (Weiser) (Nematoda: Steinernematidae) against this weevil in a semi-field trial including both preventative and curative assays has been studied. Our results prove the potential of this nematode to control *R. ferrugineus*. Efficacies around 80 % were obtained in the curative assay, and up to 98 % in the preventative treatment. Applications repeated every 2-3 weeks during the flight critical periods could prove effective to protect palms from this weevil in the Mediterranean Basin.

Keywords: Curculionidae, Steinernematidae, Palms, EPNs

Introduction

The red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera, Curculionidae), is a phytophagous insect that feeds in soft succulent tissues of many palm species (Murphy and Briscoe, 1999). Females deposit their eggs in separate holes or injuries at the base of the fronds. Eggs hatch into legless larvae which bore into the interior of the palms. On completion of their development, larvae move back to the base of the fronds where they pupate in elongate oval, cylindrical cocoons made out of fibrous strands. The red palm weevil is a concealed tissue borer and can spend all of its life stages inside the palm. Adults often remain and reproduce within the same host until the apical growing area of the palm has been destroyed by the larvae causing the palm to die. The complete life cycle of the weevil, from egg to adult emergence, takes an average of 82 days (OEPP/EPPO 2008).

Nowadays *R. ferrugineus* is considered the main pest of palms in the Mediterranean Basin (OEPP/EPPO 2008). This pest, which is native of South and Southeast Asia (Faleiro 2006) was first detected in Egypt in date palms, *Phoenix dactylifera* L., in 1992 (Cox 1993). In Spain *R. ferrugineus* was detected in 1995 (Barranco *et al.* 1995) but remained confined in a small area in Southern Spain until 2004, when the pest appeared in different foci along the Spanish Mediterranean coast. In 2006 it was reported in the Canary Islands and today it can be found in almost all Mediterranean countries. In all these areas, *R. ferrugineus* constitutes a severe pest of the Canary palm, *Phoenix canariensis* (Chabaud), which is an important ornamental plant in both public and private gardens. Furthermore, the original wild populations of this species located in the palm forests existing in the Canary Islands are presently at risk.

The methods currently used to control *R. ferrugineus* are mainly based on the application of large quantities of synthetic chemical insecticides although there are

deep concerns about the environmental pollution caused by these treatments (Faleiro 2006). Insecticides are applied in a range of preventative and curative procedures designed to limit and contain the spread of an infestation. These procedures have been developed and refined since commencing in India in the 1970s (Murphy and Briscoe 1999). Methods range from general dusting of the leaf axils after pruning, or spraying of the tree trunk, to localized direct injections of chemicals into the trunk (Faleiro 2006). Researchers have concluded that because of the cryptic habitat of the boring stages of this weevil, chemical insecticides have to be applied frequently and over a long period of time for effective management of established populations (Murphy and Briscoe 1999; Ferry and Gómez 2002).

The use of entomopathogenic nematodes (EPNs) could offer an interesting alternative to the chemical control of *R. ferrugineus* (Abbas *et al*. 2001a,b; Elawad *et al.* 2007; Saleh and Alheji 2003). EPNs are safe for non-target vertebrates and to the environment and since they are mass produced in liquid media, production costs have been significantly reduced in recent times (Ehlers 2003). Steinernematids are soil-inhabitating EPNs, and have free-living, parasitic and saprophytic stages (Mrácek 2003). The infective third juvenile stages (Dauer Juvenile, DJ) survive outside the insect and can either actively search for hosts (cruisers) or wait for host to pass by (ambushers). DJs enter the insect host through any opening (mouth, anus, spiracles) and grow into the parasitic stage. The death of the insect due to nematode parasitism is caused by Gram-negative bacteria which are carried within the gut of the DJs (Forst and Clarke 2002). *Steinernema carpocapsae* (Weiser) (Nematoda: Steinernematidae), which is mutualistically associated with the bacterium *Xenorhabdus nematophila* (Enterobacteraceae), is the most studied, available, and versatile of all EPN. This species is a typical ambusher, standing on its tail in an upright position near the soil surface (nictating) and attaching

to passing hosts. Consequently, *S. carpocapsae* tends to be most effective when applied against highly mobile surface-adapted insects (García del Pino 2006; Gaugler 2007). Cryptic habitats are considered as the most favorable, enhancing the infectivity, survival, and persistence of EPNs, because these environments minimize nematode death from ultraviolet radiation and desiccation (Mrácek 2003). Till present, field experiments in these habitats have provided mostly consistent and efficient results. *Steinernema carpocapsae* has proved successful in the control of geophilous insects, such as *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae) (Martínez de Altube *et al*. 2007), *Popilia japonica* Newman (Coleoptera: Scarabaeidae) (Simões *et al.* 1993), *Delia radicum* (L.) (Diptera: Anthomyiidae) (Schroeder *et al.* 1996), as well as species with partially hidden life cycles, such as borers, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) (Ben-Yakir *et al.* 1998), and leafminers, *Liriomyza trifolii* Burgess (Diptera: Agromyzidae) (Hara *et al.* 1993). *Steinernema carpocapsae* has already been used against *R. ferrugineus*. In the laboratory, results were good, but inconsistent in the field when used in date palms (Abbas *et al.*, 2001b). The objective of this study was to test the efficacy of *S. carpocapsae* against *R. ferrugineus* in a semi-field trial including preventative and curative assays.

Materials and methods

The assays reported in this study were carried out at the Institut Valencià d'Investigacions Agràries (IVIA) during the months of June, July and August 2007. Trials were performed in a double mesh security enclosure containing 24 independent cages (4 * 3 * 3 m) under natural light and temperature conditions. Mean temperature during the assays was 28.2ºC (max: 34.2ºC; min: 22.3ºC). A plastic roof protected the enclosure from the rain.

Plant material. Trials were performed on 48 4-year old potted *P. canariensis*. The stipe of these palms was 0.35 to 0.55 m high and 0.3 to 0.5 m wide. Plants were watered twice a week.

Experimental insects. Adult weevils used to infest the palms in the preventative tests were captured in the province of Valencia in traps baited with ferrugineol (male RPW aggregation pheromone) and plant kairomones (ethyl acetate, pieces of palm leaves). Before release, adults were kept for 3 d in a plastic lunchbox with a perforated lid where thin apple slices were provided as food source.

Immature stages used in curative tests were obtained from an artificial rearing maintained at IVIA. Eggs were obtained from wild specimens kept in plastic boxes as above and offered thin apple slices both as food and oviposition substrate as described by Martín and Cabello (2006). Eggs were subsequently transferred to an artificial complex diet (Martín and Cabello 2006) until they reached the desired age.

Nematode application. The commercial product Biorend R Palmeras® consisting of *S. carpocapsae* with a chitosan adjuvant was used. Chitosan is an organic biodegradable product with the active ingredient N-acetyl-glucosamine. The use of nematodes with chitosan is patented and has already been used in other systems (Martínez de Altube *et al.* 2007). Product was applied with a manually operated backpack compact sprayer at a dose of $1.8 * 10^6$ DJs + 18 ml chitosan per liter of water. Approximately 2 l of this solution were applied on the trunk and the bases of the fronds of each palm until run-off.

Curative tests. Twenty four palms were infested with nine larvae of *R. ferrugineus* each. A hole 3 cm deep and 1 cm in diameter was drilled on one side of the palm. Subsequently, an open vial containing the insects's artificial diet and four 7-d old larvae was introduced into the hole. Two days later the vial was removed and checked under

microscope for evidence of larval exit. Ten days later, a new hole was drilled on the other side of the palm trunk and five 15-d old larvae were similarly introduced in each palm. One month after the first infestation, twelve palms were treated with *S. carpocapsae*, and the remaining 12 were used as control. Fourteen days after the treatment 4 control and 4 treated palms were carefully dissected and checked for the presence of *R. ferrugineus*. The remaining 16 plants were similarly dissected two weeks later. All *R. ferrugineus* specimens found, either dead or alive, were counted. To ascertain whether the nematode was the cause of the death, dead immature stages were observed under microscope and streaked on NBTA (Nutrient agar supplemented with bromothymol blue and triphenyl-tetrazolium chloride) plates (Akhurst 1980) to verify the presence of *X. nematophila* phase I which absorbs bromothymol blue, producing dark blue colonies (Chavarría-Hernández *et al.* 2007).

Preventative tests. Twelve uninfested palms were treated with *S. carpocapsae* as described above and 12 additional palms constituted the control treatment. Immediately after the treatment, four control palms and four treated palms were infested with *R. ferrugineus* by releasing 4 adult presumably-mated females per plant. To evaluate product persistence the same procedure was repeated 15 and 30 days after the treatment. One week after their release, when found, females were removed from the cage. One month after the release, palms were carefully dissected and checked for the presence of *R. ferrugineus* larvae. All specimens found, either dead or alive, were counted and checked for presence of *X. nematophila* as above.

Data analysis. Results (percentage mortality and number of immature stages found alive for the curative and preventative tests, respectively) were subjected to a two-way-analysis of variance (ANOVA, $P > 0.05$). The two factors were time and treatment. The efficacy of treatments was evaluated according to Abbott (1925).

Results

Both the treatment and the time until palm dissection significantly affected the mortality caused by *S. carpocapsae* in *R. ferrugineus* when used as a curative treatment (Table 1). The longer the time elapsed since EPN application, the higher the mortality observed and hence the efficacy of the treatment. A high percentage (77.1%) of the grubs found dead in treated palms 14 d after treatment proved positive for the presence of either nematodes or the symbiotic bacterium *X. nematophila*. However, this figure dropped to just 30.2 % 14 d later. One difficulty encountered during the execution of this assay was the impossibility of recovering the nine larvae inoculated at the beginning of the assay in the treated palms. Only one to four individuals could be identified 28 d after the treatment. Around 40% of the immature stages found dead in this assay corresponded to pupae and the remaining 60% were larvae.

In the case of the preventative treatment, both the application of *S. carpocapsae* and the timing of this application in relation to infestation significantly affected the number of immature stages found alive in the palms. The interaction between these factors was significant and the number of living immature stages per palm remained low and did not significantly change from 0 to 15 d, but increased at 30 d. As a consequence efficacy decreased at the end of the assay (Table 2). Females of *R. ferrugineus* released for oviposition at the beginning of this assay were able to cause infestation in all control palms. On the contrary, the *S. carpocapsae* treatment prevented infestation in 75% of the palms for releases made either 0 or 15 d after nematode application. This result could be explained by the fact that around 50% of ovipositing females released for infestation on treated palms were found dead at the base of the fronds when the palms were dissected at the end of the assay. These fronds showed feeding holes made by

these females, but no evidence of larval galleries. However, because of the long time elapsed since their death, it was not possible to prove the involvement of *S. carpocapsae*. This was not the case of 60% of the females recovered from treated palms one week after their release which proved infected by *S. carpocapsae* when examined under binocular microscope.

Discussion

Our results prove the potential of *S. carpocapsae* to control *R. ferrugineus* infestations in palms. The dose used in our assays ($3.6* 10^6$ DJs per palm) is higher than the dose used by Abbas *et al.* (2001b) in a field assay in date palms (approximately $0.9 * 10^6$ DJs per palm) and this may partly explain the lack of effect reported by these authors. Contrarily, our dose is similar to that used by Dillon et al. (2006) against *Hylobius abietis* (L.) (Coleoptera: Curculionidae) on pine stumps (*Pinus contorta* Dougl. ex Loud and *Pinus sylvestris* L.) ($3.5 * 10^6$ DJs/tree), where efficacies of up to 47 % were reported.

Efficacies around 80 % were obtained in the curative assay, and up to 98 % in the preventative treatment. These efficacies are very high, especially when compared to chemical pesticides used against this pest (Azam and Razvi 2001; Hernández-Marante *et al.* 2003; El-Sabaey 2004; Kaakeh 2006). Furthermore, our results demonstrate the ability of *S. carpocapsae* to infect and kill not only larvae but also more robust stages of this weevil, such as the pupa and the adult. Whether infected adults could contribute to the natural spreading of this EPN within palm groves or gardens deserves further investigation.

The results from the curative assay are indicative that *S. carpocapsae* does not stay on the outside of the palm waiting for its host, but rather penetrates in the palm crown actively looking for and infecting *R. ferrugineus* larvae. These results differ from the

general consensus that this species is a classic ambusher (García del Pino 2006; Gaugler 2007), but are in agreement with results on similar cryptic systems (Dillon et al. 2006). These authors presented evidence that *S. carpocapsae* did not simply remain on the treated surface and behaved in the same way as other EPN with different foraging strategies. Similarly to our results, these authors also found that the percentage of insects parasitized by *S. carpocapsae* increased between the 2 and 4 weeks and they attributed this fact both to the time taken by the nematodes to find the insects and that taken by the insects to die after EPN infection.

The percentage of grubs found dead in treated palms which proved positive for the presence of either nematodes or the symbiotic bacterium decreased from 14 to 28 d. This result is in agreement with the hypothesis that EPNs leave their hosts once they are dead and thus contribute to the natural spread of the disease (Ehlers 2001). The same hypothesis could explain why it was not possible to isolate *S. carpocapsae* from the females found dead when the palms from the preventative treatment were dissected at the end of the assay. Besides, the ability of EPNs to decompose their hosts (Chavarría-Hernández *et al.* 2007) could account for the results obtained in treated palms whereby on completion of its infectious cycle, host larvae could no longer be recognized.

Rhynchophorus ferrugineus presents in the Mediterranean two main flight periods: around April-May and in September-October. Open field applications of EPN timed slightly before these months could protect the palms by affecting (1) immature stages from the old generation within the palm, (2) adults before oviposition and (3) young larvae from the new generation. Because the results obtained in the preventative assay prove that *S. carpocapsae* can survive in the palm for at least two weeks without loosing its efficacy, applications repeated every 2-3 weeks during these critical periods could prove effective to protect palms from this weevil.

Acknowledgments

The authors thank Alberto Urbaneja for critically reviewing an earlier version of this paper and the palm nurserymen association ASFPLANT for providing the palms used in these assays. This research was partly funded by the INIA (project TRT2006-00016-C07-05) and IVIA (project 5611).

References

Abbas MST, Hanounik SB, Mousa SA and Mansour MI (2001a) On the pathogenicity of *Steinernema abbasi* and *Heterorhabditis indicus* isolated from adult *Rhynchophorus ferrugineus*. Inter J Nematol 11: 69–72.

Abbas MST, Saleh MME and Akil AM (2001b) Laboratory and field evaluation of the pathogenicity of entomopathogenic nematodes to the red palm weevil, *Rhynchophorus ferrugineus* (Oliv.)(Col.:Curculionidae). J Pest Science 74: 167-168.

Abbott WS (1925) A method of computing the effectiveness of an insecticide. J Econ Entomol 18: 265-267.

Akhurst RJ (1980) Morphological and functional dimorphism in *Xenohabdus* spp., bacteria symbiotically associated whit the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. J Gen Microbiol 121: 303–309.

Azam KM and SA Razvi (2001) Control of red palm weevil, *Rhynchophorus ferruginous* Oliver by prophylactic spraying of date palms and trunk injection. Proc 2nd Int Conf Date Palm, Al-Ain, UAE: 216-222.

Barranco P, De la Peña J and Cabello T (1995) Un nuevo curculiónido tropical para la fauna europea, *Rhynchophorus ferrugineus* (Olivier 1790) (Coleoptera, Curculionidae). Bol Asoc Esp Ent 20: 257-258.

Ben-Yakir D, Efron D, Chen M and Glazer I (1998) Evaluation of entomopathogenic nematodes for biocontrol of the European corn borer, *Ostrinia nubilalis*, on sweet corn in Israel. Phytoparasitica 26: 101-108.

Chavarría-Hernández N, Sanjuan-Galindo R, Rodríguez-Pastrana BR, Medina-Torres L and Rodríguez-Hernández AI (2007) Submerged monoxenic culture of the

entomopathogenic nematode *Steinernema carpocapsae* in an internal-loop airlift bioreactor using two configurations of the inner tube. Biotechnol Bioengin 98:167-176.

Cox ML (1993) Red palm weevil, *Rhynchophorus ferrugineus,* in Egypt. FAO Plant Prot Bull 41: 30-31.

Dillon AB, Ward D, Downes MJ and Griffin CT (2006) Suppression of the large pine weevil *Hylobius abietis* (L.) (Coleoptera: Curculionidae) in pine stumps by entomopathogenic nematodes with different foraging strategies. Biol Control 38: 217-226.

Ehlers RU (2001) Mass production of entomopathogenic nematodes for plant protection. Appl Microbiol Biotechnol 56: 623–633.

Ehlers RU (2003) Entomopathogenic nematodes in the European biocontrol market. Commun Agric Appl Biol Sci 68: 3-16

Elawad SA, Mousa SA, Shahdad AS, Alawaash SA and Alamiri AMA (2007) Efficacy of entomopathogenic nematodes against red palm weevil in UAE. Acta Horticulturae, 736: 415-420.

El-Sabaey (2004) Field evaluation of certain insecticides against red palm weevil *Rhynchophorus ferrugineus* Oliv. (Coleoptera; Curculionidae) in Egypt. Egypt J Agric Res 82: 1591-1598.

Faleiro JR (2006) A review of the issues and management of the red palm weevil *Rhynchophorus ferrugineus* (Coleoptera: Rhynchophoridae) in coconut and date palm during the last one hundred years. Int J Trop Insect Sci 26: 135-154.

Ferry M and Gómez S (2002) The Red Palm Weevil in the Mediterranean Area. Palms 46**:** 172-178.

Forst S and Clarke D (2002) Bacteria-nematode symbiosis. In: Gaugler R. (ed). Entomopathogenic nematology. Cambridge MA, USA.

García del Pino F (2006) Los nematodos entomopatógenos agentes de control de plagas. In: Jacas J Caballero P and Avilla J. (eds) El control biológico de plagas y enfermedades. Publicacions de la Universitat Jaume I. Castelló de la Plana. Spain.

Gaugler R (2007). Nematodes (Rhabditida: Steinernematidae & Heterorhabditidae). http://www.nysaes.cornell.edu/ent/biocontrol/ accessed. Cited 25_Jul_2008.

Hara AH, Kaya HK, Gaugler R, Lebeck LM and Mello CL (1993). Entomopathogenic nematodes for biological control of the leafminer, *Liriomyza trifolii* (Dipt.: Agromyziidae). BioControl 38: 359-369.

Hernández-Marante D, Folk F, Sánchez A, Fernández-Escobar R. (2003). Control del curculiónido ferruginoso de las palmeras (*Rhynchophorus ferrugineus* Olivier) mediante inyecciones al tronco y pulverización foliar. Bol. de San. Veg. Plagas, 29: 563-574.

Kaakeh, W (2006). Toxicity of imidacloprid to developmental stages of *Rhynchophorus ferrugineus* (Curculionidae: Coleoptera): Laboratory and field tests. Crop Prot 25: 432-439.

Martín MM and Cabello T (2006) Manejo de la cría del picudo rojo de la palmera, *Rhynchophorus ferrugineus* (Olivier, 1790) (Coleoptera, Dryophthoridae), en dieta artificial y efectos en su biometría y biología. Bol. San. Veg. Plagas, 32: 631-641.

Martínez de Altube MM, Strauch O, Fernández De Castro G and Martínez Peña A (2007). Control of the flat-headed root borer *Capnodis tenebrionis* (Linné)

(Coleoptera: Buprestidae) with the entomopathogenic nematode *Steinernema carpocapsae* (Weiser) (Nematoda: Steinernematidae) in a chitosan formulation in apricot orchards. BioControl 53: 531-539.

Mrácek Z (2003) Use of entomopathogenic nematode EPANs in Biological Control. In: Upadhyay RK (ed). Advances in Microbial Control of Insect Pests. Plenum Pub Corp. New York. USA.

Murphy ST and BR Briscoe (1999). The red palm weevil as an alien invasive: biology and the prospects for biological control as a component of IPM. BioControl. News & Information, 20: 35-46.

OEPP/EPPO (2008). Data sheets on quarantine pests. *Rhynchophorus ferrugineus.* Bull. OEPP/EPPO 38: 55-59.

Saleh MME and Alheji M (2003) Biological control of red palm weevil with entomopathogenic nematodes en the eastern province of Saudi Arabia. Egypt J Biol Pest Control, 13: 55-59.

Schroeder PC, Ferguson CS, Shelton AM, Wilsey WT, Hoffmann MP and Petzoldt C. (1996) Greenhouse and field evaluations of entomopathogenic nematodes (Nematoda: Heterorhabditidae and Steinernematidae) for control of cabbage maggot (Diptera: Anthomyiidae) on cabbage. J Econ Entomol 89: 1109-1115.

Simões N, Laumond C and Bonifassi E (1993) Effectiveness of *Steinernema* spp. and *Heterorhabditis bacteriophora* against *Popillia japonica* in the Azores. J Nematol 25: 480-485.

Table 1. Results of the curative assay: mean number of immature stages of *R. ferrugineus* found in *P. canariensis* 14 and 28 days after EPN application (3.6 * 10^6 DJs + 36 ml chitosan per palm), mortality and efficacy of each treatment. (Initial infestation was 9 larvae introduced 1 month before treatment)

Treatment	Time (d) after treatment application	n	Mean number of immature stages (±SE)		Mortality (%)	Efficacy (%)
			Alive	Dead		
Control	14	4	5.50 ± 1.11	0	0	
EPN		4	1.25 ± 0.29	3.25 ± 0.29	72.9 ± 2.4	72.9 ± 2.4
Control	28	8	5.13 ± 0.62	0.13 ± 0.13	2.5 ± 2.6	80.8 ± 10.6
EPN		8	0.50 ± 0.29	2.00 ± 0.45	81.3 ± 10.3	
ANOVA results*						
Source of variation			*F*	*df*		*P*
Treatment			163.28	1, 20		< 0.0001
Time			5.62	1, 20		0.0298
Interaction			3.10	1, 20		0.0962

Data subjected to a two-way-analysis of variance (ANOVA $P > 0.05$).
*Data subjected to the angular transformation prior to analysis.

Table 2. Results of the preventative assay: mean number of immature stages of *R. ferrugineus* found in *P. canariensis* as a function of the time elapsed since EPN application (0, 15 and 30 d; dose 3.6 * 10^6 DJs + 36 ml chitosan per palm) and efficacy of each treatment. Palms were infested with 4 mated females each and dissection took place 1 month after infestation.

Treatment	Time (d) after treatment application	n	Mean number of immature stages (±SE)		Efficacy (%)
			Alive	Dead	
Control	0	4	23.00 ± 3.50	0	93.5 ± 7.5
EPN		4	1.50 ± 1.73	0.75 ± 0.87	
Control	15	4	14.25 ± 7.53	0.25 ± 0.29	98.2 ± 2.0
EPN		4	0.25 ± 0.29	0	
Control	30	4	83.50 ± 11.13	2.00 ± 1.56	75.7 ± 8.1
EPN		4	20.25 ± 6.79	0	

ANOVA results*

Source of variation	*F*	*df*	*P*
Treatment	53.72	1, 23	< 0.0001
Time	39.52	2, 23	< 0.0001
Interaction	11.64	2, 23	0.0006

Data subjected to a two-way-analysis of variance (ANOVA P>0.005).

*Data subjected to the *log (x+1)* transformation prior to analysis.