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SCANNING ELECTRON MICROSCOPIC CHANGES IN *HABRONEMA MUSCAE* AFTER IN VITRO EXPOSURE TO PLANT EXTRACT (*VERBESINA ALTERNIFOLIA*)

By

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Abstract

Scanning electron microscopy (SEM) was used to study the morphological alteration occurring in *Habronema muscae* adult female worms after in vitro exposure to different doses of *Verbesina alternifolia* oil extract. The half maximal lethal concentration (LC_{50}) was reached 400 ppm after 24hrs, while LC_{100} was reached 600 ppm after 48hrs. Irreversible degenerative changes were recorded such as shrinking, detachment and distortion of the cuticle, cephalic and distal region. The cuticular surface had a wrinkled, corrugated appearance with longitudinal ridges and transverse thick folds. The lips and papillae were deformed and aggregated over each other. There is a direct relation between the level of the recorded degenerative changes and the increase in the dose and exposure time. In the same time no degenerative changes were recorded in the control worm exposed to PBS till the end of the exposure period.

Key words: *Verbesina alternifolia, Habronema muscae,* SEM, in vitro study

Introduction

Twelve *Habronema* species in mammals, mostly equids were listed causing gastric, cutaneous and conjunctival habronemiasis (Gasthuys *et al,* 2004). *Habronema muscae* is a parasite of horses, ponies, donkeys and zebras. The adult worms live superficially, under the mucous layer, in the stomach with only their heads buried in the mucosal spaces. When the larvae are licked and swallowed by the horse during grooming they travel to the stomach and embed themselves into the glandular part of the stomach close to the margo plicatus. A thick mucus is excreted by the stomach lining. The larvae mature into adults and females produce eggs to complete the life cycle. Larvae that invade skin or eye tissue do not develop into adults (Soulsby, 1986).

Habronema infection in stomach stimulates the secretion of large amounts of thick, stringy, viscous mucus, associated with hyper-plasia and hypertrophy of the stomach mucus-secreting cells. If the larvae travel through the nose, into the pulmonary system and then the lungs, they can cause abscesses in the lung tissue where they imbed themselves in the lungs tissue (Naem, 2007). Also, catarrhal gastritis, ulcers, diarrhea and weight loss have been reported (Traversa *et al,* 2004)\

Synthetic anthelmintics alone cannot combat *Habronema* spp. infection. The current state of crisis regarding widespread anthelmintic resistance calls for alternative control strategies. To circumvent the problem of drug resistance, the only realistic strategy would be to develop novel non-chemical approaches that decrease the need of treatment. Moreover, the use of the anthelmintics proved effective in a more intelligent manner (Sanyal, 2005). Phytomedicine has been used to treat parasitism. Moreover; many modern commercial medicines are derived from plants. However, scientific evidence on the anti-parasitic efficacy of most plant products were limited (Githiori *et al,* 2006).

The present work aimed to evaluate anthelmintic properties of *Verbesina alternifolia* (Crown Beard) versus *H. muscae* by Scan Electric Microscopy.

Materials and methods

This study was designed and approved by Faculty of Veterinary Medicine Cairo University Ethics Committee and has been performed in accordance with the ethical standards laid down in the 1964 Declaration of

Helsinki. This study was conducted between March to June 2015.

Collection of adult *H. muscae*: Female worms used in the in vitro trials were collected from two naturally infected horses humanely slaughtered in the main slaughterhouse of Giza Zoo. The stomach of each horse was obtained immediately and transferred to the laboratory where it was opened in order to collect the female adult parasites using a fine needle.

Production of the plant extracts: *Verbesina alternifolia*, oil extract was kindly obtained from Dr. M.M. Salama (Dept. of pharmacognosy, Faculty of pharmacy, Cairo Univ.). It was prepared according to its previous description (Salama *et al*, 2012).

In vitro evaluation of the plant extract on *Habronema muscae*: Efficacy of plant extracts was evaluated using series of increasing concentrations (100, 200, 400 & 600 ppm) each for 6, 12, 24 & 48 hours exposure time. Each experiment set contained two replicates each of 10 worms. Control group in solvent as well as in PBS was associated with each exposure time. The bioassay was done according to WHO (1996) guidelines. Fresh active clean motile worms were selected for the experiment. All experiments were conducted simultaneously in the laboratory in an incubator at 37ºC. At the end of the exposure time, tested solution was removed. Worms were washed for five times using PBS solution, left for another 3 hours, mortality rate (%) were evaluated after counting the number of dead worms to the total number of the exposed worms (Taher *et al*, 2012).

Samples from recently dead and alive worms were collected and fixed in 1.5ml eppendorf tubes with Glutraldhyde 2.5% for SEM examination and photographing.

Procedure of scanning electron microscopy (SEM): The worms obtained from the in vitro assays including the untreated control group were fixed in a 2.5% glutraldhyde solution in a 0.1 M sodium cacodylate buffer for 4 hrs. at 4ºC. After two washes in the same buffer (0.2 M), the worms were dehydrated in a graded ethanol series, dried by critical point drying with EMSCOPE CPD 750 and coated with gold–palladium for 5 min at 100 min-1. Parasites were examined with a S450 SEM (Hitachi) at an accelerating voltage of 15 kV at Faculty of Agriculture, Cairo University.

Results

Exposure of female *H. muscae* worms to *V. alternifolia* fixed oil extract caused mortalities associated with irreversible degenerative changes in the cuticle, anterior, posterior and sensitive papillae on exposed body. The degenerative effect increased with the increase in the dose and exposure time to the extract. The mean LC_{50} and LC_{100} of the tested oil extract were reached at 400ppm after24hrs and at 600ppm after 48hrs respectively. No mortality was recorded in worms exposed for the same periods in PBS as control.

Dead worms exposed to *V. alternifolia* oil at 600ppm after 48 hrs appeared shrinked, corrugated cuticle with more or less detachment of epicuticular layers (Fig.1) marked degenerative changes in body (cuticle) and cephalic region.

The group treated with *V. alternifolia* lost normal aspect, with clear distortion of both buccal capsule and cuticle, lips were deformed and aggregated in and around buccal capsule (Fig1.b). The cuticular surface had a wrinkled, corrugated appearance with longitudinal ridges and transverse thicker folds in the cuticle, observed either on the body totality or patches along the entire body including the cephalic parts (Fig. 2) and rest of body as well as distal portion. The obvious changes were the aggregates that located in and around buccal capsule, and controls in PBS were active with smooth cuticle without any degree of degeneration (Fig.1a)

Concerning the effect of *Verbesina* oil extract on ciliated cervical papilae at cervical regions of the exposed worms showed aggregation to these papillae and in the same time the cuticle lost their smooth appearances due to the presence of great longitudinal

ridges (Fig.3b). While the controls appeared normal with smooth cuticle and normal transverse striation (Fig. 3a). Concerning the degenerative changes recorded in distal end of treated females (Fig. 4) showed wrinkling of cuticular ridges along entire worms when compared with controls (Fig.4a).

Discussion

Nowadays several plants from different families have been reported for their anthelmintic activity as *Sphaeranthus indicus* Linn. in India (Ramachandran, 2013).

Only few botanicals have moved from the laboratory to field use (Githiori *et al,* 2006). The seeds in a fixed oil had anthelmintic, in its extracts as *Madia sativa*, *Verbesina sativa* (Lindley, 2013). The main overall effect was a significant reduction in egg excretion which reached -80% from the controls in some studies (Lange *et al,* 2006; Manolaraki *et al,* 2010), related either to a reduction in the worm number (Heckendorn *et al,* 2006; Terrill *et al,* 2007; 2009) and/or to a reduced fertility per female when this parameter was measured (Lange *et al,* 2006; Heckendorn *et al,* 2007; Manolaraki *et al,* 2010). In Egypt, *Verbesina* alcoholic extracts examined for molluscicidal and mosquito larvicidal activities and the major poisoning effect corresponded to high levels of nitrates and galegine (Taher *et al,* 2012) and due to phyto-constituents of *Verbesina* as terpenoids, flavonoids and aromatic compounds (Song *et al,* 2009; Sindhu *et al,* 2010)

The results revealed that the parasites treated with plant extracts lost their normal aspect by showing longitudinal folds and thicker ridges in the cuticle compared with the control worms. The most striking changes observed as aggregates located in and around the buccal capsule that revealed the presence of adulticidal effect of alcoholic extracts might be attributed to the presence of fixed oil present in these plant extracts. Also, the cuticle of nematodes is metabolically active and morphologically specialized for selective absorption of nutrients and osmoregulation (Alvarez *et al,* 2007). Thus, passive diffusion of anthelmintic through the cuticle would probably be responsible for destructive changes and deformation of nematode body surface (Tippawangkosol *et al,* 2004; Schmahl *et al,* 2007). In general, cuticle of nematodes was known to be the basic entry route and primary site of activity of anthelmintic drugs (Alvarez *et al,* 2007)

The anthelmintic activity of plant extracts was reported by many workers whose results comply with present study as Akhtar *et al.* (1999) who studied *Chenopodium album* against nematodes in sheep. Mantawy *et al.* (2012) found antioxidant and schistosomicidal effect of *Allium sativum* and *A. cepa* against different stages of *Schistosoma mansoni*. Bazh and El-Bahy (2013) In vitro and in vivo found that ginger and curcumin extracts had potential anthelmintic properties against *Ascaridia galli* and that ginger in all concentrations used exhibited a higher death rate observed than curcumin. Apart from helminthes, Massoud *et al.* (2008) successfully used *Commiphora molmol* or combined with paromomycin in treating cryptosporidiosis *parvum* in immunocompetent hospitalized patients; Abdel Hady *et al.* (2008) successfully treatment of *Toxoplasma gonii* by two Egyptian herbs and El-Sherbini *et al.* (2009) in-vitro and in-vivo control successfully treated *Trichomonas vaginalis* with *Punica granatum* extract. Abouel-Nour *et al.* (2016) reported that cryptosporidiosis *parvum* infected mice were treated with three Egyptian medicinal plants; ginger, mirazid, garlic as compared to metronidazole significant symptomatic improvements during treatment.

On the other hand, it can be speculated that the presence of aggregates around the anterior part of the digestive tract (i.e., buccal capsule) may disturb the nematodes nutrition, which might eventually lead to worm undernourishment, reduced fertility and/or mortality. The potential presence of aggregates in the cephalic part of *H. muscae* might disturb the mechanical and/or enzymatic processes which are normally in-

volved in the consumption of blood meals by the worms (Fetterer and Rhoads, 1997). Last, the structural changes in the external reproductive organs of female worms treated with *Verbesina alternifolia* extract showed obvious wrinkling in their cuticles) could affect the nematode reproductive function.

Also, the results agreed with Youssif and Shaalan (2011) who reported that the free using of commercially purified, extraction of botanical active component proved to be a new field of preparation of safe, rapidly biodegradable and eco-friendly materials alternative to chemical ones. *Verbesina alternifolia* oil extract as Egyptian native plants continue to provide a wealth of potential sources for biologically active agents that may be applied against some parasitic stages of man and animals.

In Egypt, Khalifa *et al.* (1988) in Assuit found *H. muscae* in 45.81% of 48 equines. by necropsy of 65 donkeys in Giza Zoo. Ahmed *et al*. (2011) detected 23.0% *Habronema* spp. The predominant one was *H. muscae* (21.54%) followed by *H. microstoma* (15.3%) and *H. megastoma* (7.69%). Apart from *Habronema* sp. Abo-Madyan *et al.* (2004) in Al-Fayoum reported that Myrrh extract of *Commiphora molmol* proved safe and effective in the treatment of human fascioliasis under field conditions. Haridy *et al*. (2006) in Gharbia successfully treated paramphistomiasis infected cows, buffaloes and sheep with Oleo-resin solution of *Commiphora molmol* (dose of 6 ml of 10gm% equal to 2 Mirazid). Eida *et al*. (2016) reported that N. sativa had inhibitory effect on *Blastocystis hominis* in vitro and prevented cytopathic effect in infected mice inoculated orally with B. hominis

Abroad, Pandey and Eysker (1988) in Zimbabwe among 14 donkeys reported *H. muscae* in 12 (85.7%). Mfitilodze and Hutchinson (1989) in northern Queensland in 57 horses reported *H. muscae* in 43%. Lyons *et al*. (1985) in Kentucky among natural infections of parasites from the lungs of 488 dead horses varied from 1 to 32 years of age; 419 horses were from 215 farms and 69 horses were from 68 individual sources for which a specific farm was not identified. Examinations of the lungs showed larvae of *Parascaris equorum* in 37 (8%) and *Habronema/Draschia* in 67 (14%) of the horses. Pandey *et al.* (1992) in Morocco and Burgu *et al.* (1995) reported higher incidences of *H. muscae* (89.7% & 100%, respectively) and *H. microstoma* (85.4% and 90%, respectively). Schuster *et al.* (2010) United Arab Emirates reported that nematode larvae in histological cuts of a horse lung tissue from a farm in Al Dhaid) were determined to belong to the Habronematidae family. They added that larvae were found in 147/561 male and 64/739 female *Musca domestica* caught at the farm. The housefly population caught in the barn showed a prevalence of 20.9% with nematode larvae, while flies trapped outside the building on the territory of the farm had a much lower prevalence of 1.1%.Larvae retrieved at the fly dissection were subjected to a ribosomal DNA-targeting semi-nested PCR protocol able to discriminate among the three nematode species *Habronema muscae, Habronema microstoma,* and *Draschia megastoma,* and identified to be *H. muscae*.

Conclusion

Undoubtedly, equines still receive much interest and care in many countries as draft animals, source of meat, leather and other related products. The results proved that *Verbesina alternifolia* oil extract has a promising role in treating *Habronema muscae*. Purified components may lead to an effective friend agent in eradication of some parasites in Egypt.

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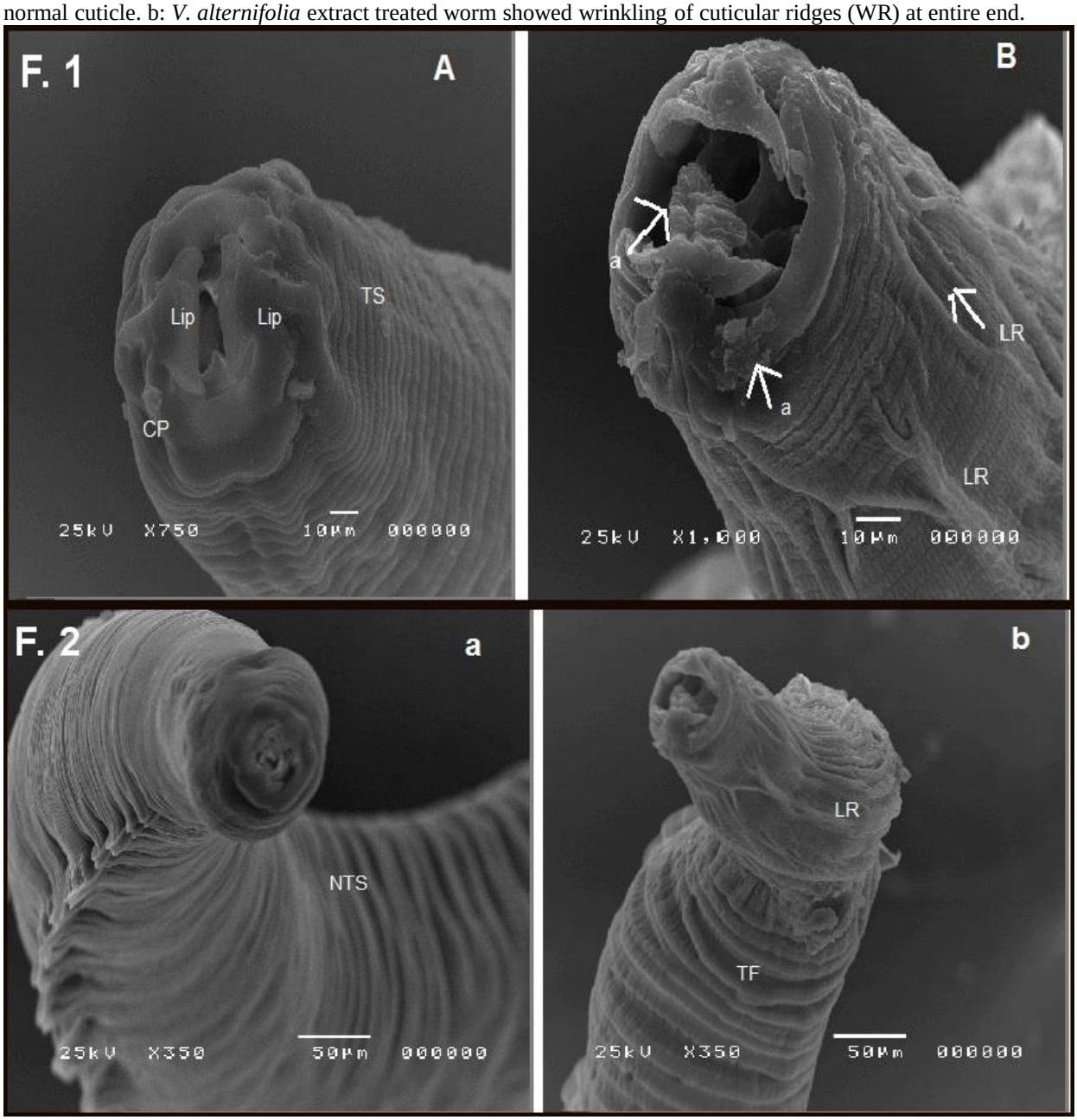
Explanation of figures

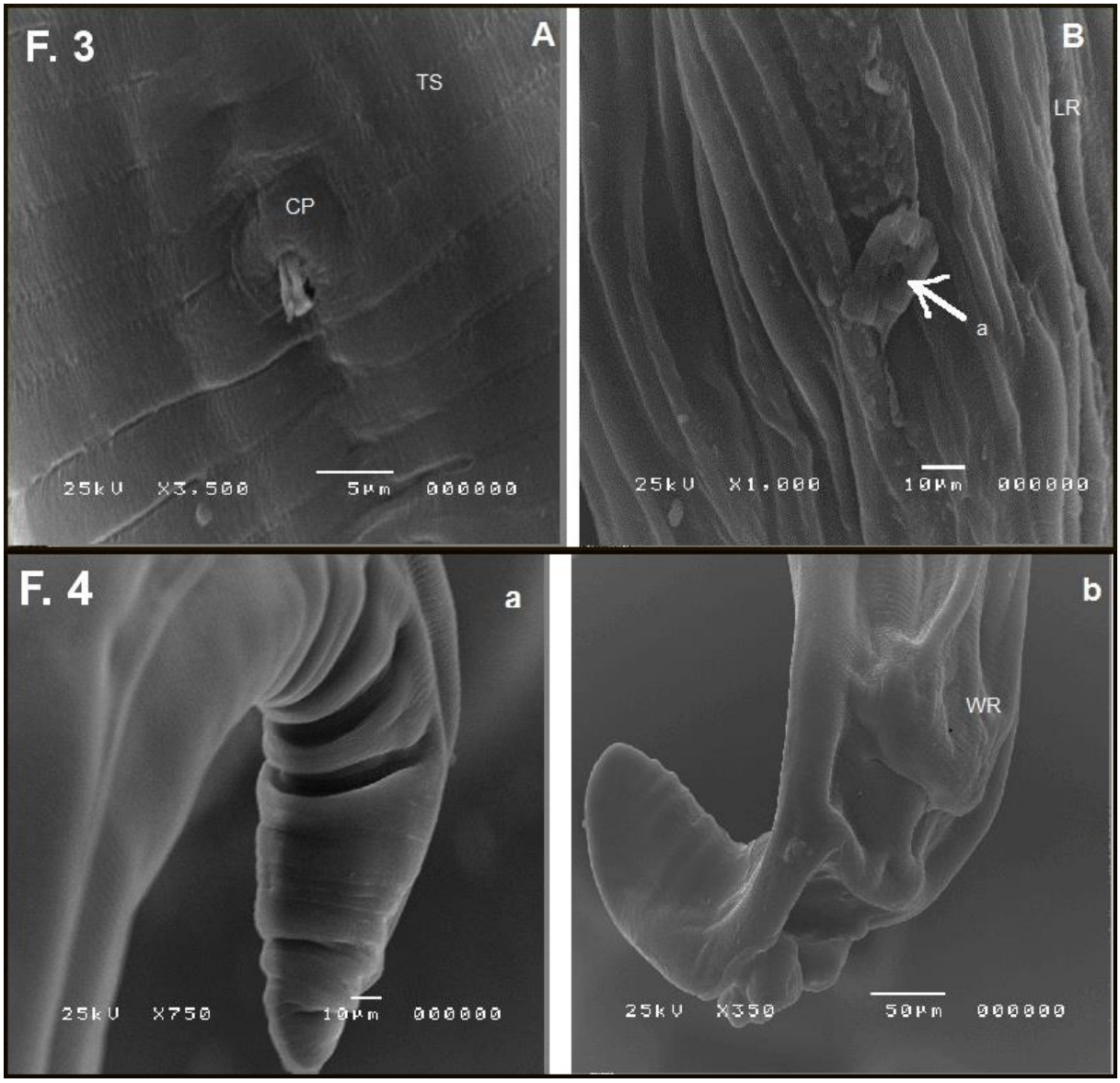
Fig.1: SEM of anterior end of female *Habronema muscae* showing cephalic region. A: control non treated worm showing two lips (Lip), sub median cephalic papillae (CP) and normal cuticular transverse striation (TS). B: *Verbesina alternifolia* extract treated worm showed distortion of two lips with accumulated aggregated (a) in and around buccal cavity, also on longitudinal ridge (LR) on worm cuticle.

Fig.2: SEM of anterior end of female *H. muscae* showing cervical region. a: control non treated worm showing normal transverse striation (NTS). b: *V. alternifolia* extract treated worm showed longitudinal ridges (LR) and transverse thicker folds (TF).

Fig.3: SEM of anterior end of female *H. muscae* showing ciliated cervical papilae. (CP). A: control non treated worm showing normal CP and cuticular striation (TS). B: *V. alternifolia* extract treated worm showed aggregates (a) covered ciliated cervical papillae and longitudinal cuticular ridges (LR).

Fig.4: SEM of anterior end of female *H. muscae* showing posterior end. a: control non treated worm showing normal cuticle. b: *V. alternifolia* extract treated worm showed wrinkling of cuticular ridges (WR) at entire end.




F. 3
A
TS
CP
25kV X3,500 5µm 000000
B
LR
a
25kV X1,000 10µm 000000
F. 4
a
25kV X750 10µm 000000
b
WR
25kV X350 50µm 000000