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Original Article

Effect of aqueous vitamin B on the growth of blister blight pathogen, *Exobasidium vexans*

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Abstract

The effect of three aqueous solution of biotin, thiamine and calcium pantothenate, on the growth of *Exobasidium vexans* was examined *in vitro*. The germination process of basidiospores of *E. vexans* differed from those of the other *Exobasidium* species. Basidiospore germination commenced after 19.5 hr incubation and chlamydospore-like bodies were formed after 96 hr of incubation. Addition of biotin, calcium pantothenate, and thiamine to Difco PDA and Czapek's medium did not affect the proportion of germinating basidiospores. The length of germ tubes was enhanced only by addition of thiamine in the media. Larger size germ tubes (thick germ tubes) were occasionally observed among the ordinary hyphae. Most germlings of basidiospores developed chlamydospore-like bodies or autolysed on the media. Thick germ tubes frequently appeared on the calcium pantothenate amended media and developed into a colony when these hyphae were transferred to new calcium pantothenate amended media. However, further transfer of colonies did not successfully bring a new colony to grow on the calcium pantothenate amended media. Vitamin B_5, calcium pantothenate, was only partially effective in generating the thick germ tubes and to induce the initial colony formation, whereas amendment of biotin and thiamine to the media did not induce visible colony growth.

Keywords: blister blight, *Camellia sinensis* var. *assamica, Camellia sinensis* var. *sinensis, Exobasidium vexans*, potato sucrose agar

1. Introduction

Exobasidium vexans Massee causes leaf blister on tea plant, *Camellia sinensis* (L.) Kuntze and significantly impacts on the cultivation of tea in Malaysia (Singh, 1980), Indonesia (Semangun, 1988), Thailand (Giatgong, 1980), the Philippines (Benigno and Quebral, 1977), and all over the world (Ezuka and Ando, 1994).

Exobasidium vexans is presumed to be an obligate parasite (Loos 1951 in Graafland, 1953) of tea and as such cannot be cultured on artificial media. Graafland (1953) first reported successful cultivation of pure cultures of *E. vexans* on maltsalep agar. It was reported that the dull spots on agar media were caused by the spore discharge of *E. vexans* and they always proved to consist of germinated basidiospores. When the basidiospores germinated, the contents of basidiospores moved into the irregularly growing germ-tubes. Young transfers also showed the typical feature of scattered half-spherical colonies and these separated colonies then formed the typical much folded colony.

Recently, several new *Exobasidium* spp. were collected in Japan (Nagao *et al.,* 2001; 2003a; 2003b; 2004a; 2004b; 2006) and more than 113 isolates of *Exobasidium* spp., except *E. vexans*, have been isolated and cultured on artificial media. Cultivation of the basidiospores of *E. vexans* has preliminarily been attempted several times on Difco-PDA and MEA. However, these attempts were unsuccessful as the basidiospores either did not germinate on the media or sometimes germinated with germ tubes but these never developed to form colonies. Location from which the basidiospores were

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collected (Ibaraki, Miyazaki and Shizuoka Prefectures) did not affect the result.

Ezuka (1955) reported successful cultivation of this fungus on artificial media. He could obtain and maintain cultures of *E. vexans* on home-made Potato Sucrose Agar (PSA), originally described as 'potato agar'. He recorded three types of germination patterns. Most basidiospores germinated but formed chlamydospore-like bodies on and/or at the end of germ-tubes. Gadd and Loos (1950) also reported chlamydospore-like bodies. Some of the basidiospores produced 'abnormally thick germ tubes' and secondary spores formed on them. These germlings developed into a yeast-like colony. *Exobasidium vexans* formed a colony after 20 days incubation on the home-made PSA.

Sundström (1964) reported that some heterotrophic *Exobasidium* strains required thiamine in order to successful cultivation. *Exobasidium angustisporium* Linder, *E. vaccinii* "vit.id" Sundström, and *E. vexans* were shown to be thiamine heterotrophs and that basidiospore formation and the ballistospore formation in the culture of *E. vexans* required supplementation of the media with 100 μg thiamine. Colonies of *E. vexans* developed after 20-30 days incubation.

Successful culturing of *E. vexans* is purported to be attributed to the presence of specific ingredient in the media. Graafland (1953) and Ezuka (1955) used media based on natural substrates, which presumably provided a broad range of micro-nutrients in the media. In contrast, Sundström (1964) used synthetic media amended with thiamine. According to the Japanese Standard Table of Food Composition (Anonymous, 2005), fresh potato and tea leaves contain several aqueous vitamin Bs, namely thiamine (B_1), niacin (B_3), pantothenate (B_5), and folic acid (B_9).

The objective in this report was to examine the effect of aqueous vitamin Bs on the colony formation and maintenance of *E. vexans*.

2. Materials and Methods

2.1 Medium

A home-made Potato Dextrose Agar (PDA) was prepared with 200 g of potato, 20 g of dextrose, and 20 g of Difco Bacto-Agar in 1000 ml of distilled water (Mueller *et al*., 2004). Sucrose was substituted with dextrose to prepare a home-made Potato Sucrose Agar (PSA). 0.1% yeast extract was amended to PSA for PSA+YE. Difco PDA, Difco Czapek Dox broth, and Malt extract agar for microbiology (Merck) were also used. Difco Bacto-Agar (15g/L) was added to Difco Czapek Dox broth in this experiment (hereafter referred as Czapek's medium).

Aqueous vitamin B solutions, viz 100 μg/L of thiamine (B_1) and 150 μg/L of calcium pantothenate (B_5), were added to Difco PDA and Czapek's medium, respectively. The concentration of each vitamin was based on those used by Sundström (1964). Biotin has not been detected in either potato or tea, but was added (10 μg/L) as biotin was added to medium 4 of Sundström (1964). Each vitamin stock solution was filtrated by NALGENE Disposable Filter unit (Pore size 0.45 μm). Hereafter, Difco PDA and Czapek's medium amended with biotin, calcium pantothenate and thiamine were abbreviated as Bio+PDA, Pan+PDA, Thiam+PDA, Bio+Cz, Pan+Cz and Thiam+Cz, respectively.

2.2 Sample of tea blister leaves

Samples of tea blister leaves were collected from *C. sinensis* var. *sinensis* at the experimental field of Shizuoka Prefectural Research Institute, Kurasawa, Kikukawa-shi, Japan, on 16 July 2003 by Mr. T. Nishijima. The others were collected from *C. sinensis* var. *assamica* in three tea plantations in Cameron Highlands (Pahang), Malaysia, on 13 December 2008.

2.3 Morphological observations

Fresh materials of *C. sinensis* var. *sinensis* in the field were used for morphological observations. Materials for morphological observations were prepared as described previously (Nagao *et al*., 2003a). The examined material was deposited in the herbarium of the Ibaraki Nature Museum, Tsukuba, Ibaraki, Japan (INM-2-052298).

2.4 Culture of basidiospores

Basidiospores were collected from fresh diseased leaves of *C. sinensis*. Leaves with lesions were cut into small pieces about 5 mm square and were fixed with about 10 mm square water agar block to the inside of the lid of a sterile Petri dish, poured with a medium acidified with 10 % (v/v) lactic acid. The dish was kept at 22ºC in the dark. Basidiospores then fell from the hymenium onto the agar surface. The process of germination of basidiospores was successively observed by light microscope as described previously (Nagao *et al*., 2003a). One hundred to150 basidiospores were observed for germination and the length of ca. 20 germ tubes were measured. The effect of vitamins on basidiospore germination was examined twice. Lacto-phenol cotton blue solution was used for staining the germ-tubes. Sudan Black B solution (1 g /L of 70% ethanol) was used to stain the lipid bodies.

2.5 Colony induction

Three colonies including chlamydospore-like bodies and/or thick germ tubes were cut from 17-days-old cultures and were further transferred to the newly prepared media, respectively. The dish was kept in the same conditions as previously described. Colony formation was observed and diameter of colony was measured under a compound microscope. Three replicates were examined, except the H-Pan+Cz (2 replicates). Colour names of colony was referred from a mycological colour chart (Rayner, 1970). Morphology of

hyphal components was examined.

3. Results

3.1 Morphological observations and culture of basidiospores

Basidia were clavate to cylindrical, 88.6-164.5 × 3.8-5.1 µm. Basidiospores were ellipsoid to ovoid, (9)11-16 × 4-6 µm, hyaline, smooth, one-septated.

Proportions of germinating basidiospores on *C. sinensis* var. *sinensis* after 19.5 hr were more than 90% on Difco PDA, Czapek's medium and home-made PSA. Addition of biotin, calcium pantothenate and thiamine to Difco PDA and Czapek's medium did not affect the proportion of germinating basidiospores (Figure 1). After 96 hr incubation, the proportions of germinating basidiospores reached close to 99%. In contrast, the proportions of germinating basidiospores after 19.5 hr were 77.6% and 64.0% on home-made PDA and PSA+YE, respectively. Germination on these two media did not increase after 19.5 hr incubation. Germination was not observed on MEA.

Germ tubes of the basidiospores emerged from each end of cell. Length of germ tubes after 19.5 hr incubation averaged more than 15 ìm except on MEA (Figure 2). Germ tubes grew straight without branching (Figure 3). After 46.5 hr incubation, chlamydospore-like bodies formed on the germ tubes except for the germlings on the home-made PDA, the home-made PSA, PSA+YE, and MEA. At 96 hr incubation, chlamydospore-like bodies were formed on the home-made PSA and PSA+YE. Autolysis of germlings was observed gradually over this period of time. On the contrary, germ tubes of basidiospores were still growing on Thiam+Cz, averaged 228.5µm length of germ tube, and Thiam+PDA, 184.3µm (Figure 2). Production of conidia on the thick germ tube were always observed on Pan+Cz (Figure 4(a)) and Thiam+Cz (Figure 4(b)), whereas the conidial production was observed only once in two times experiments on Bio+Cz (Figure 4(c)) and on Pan+PDA. Some germlings stayed in the intermediate condition between chlamydospore-like bodies and hypha (hereafter referred as the condensed hyphae). Sudan Black

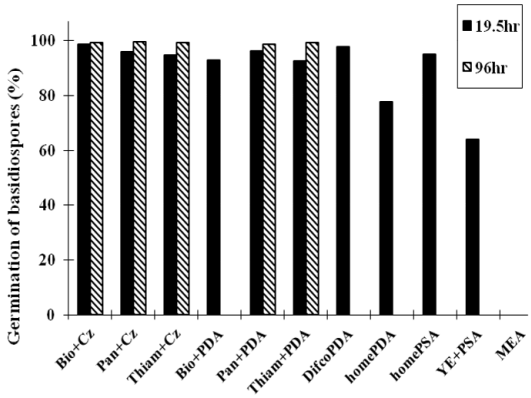

Figure 1. Percentage of basidiospore germination of *Exobasidium vexans* on 11 media.

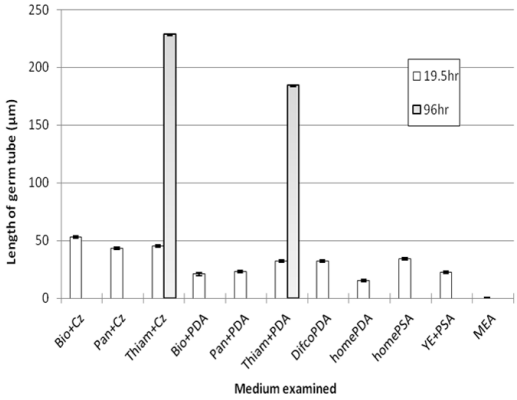

Figure 2. Length of germ tube of the basidiospore of *Exobasidium vexans* on 11 media. Error bars indicated ± one standard deviation.

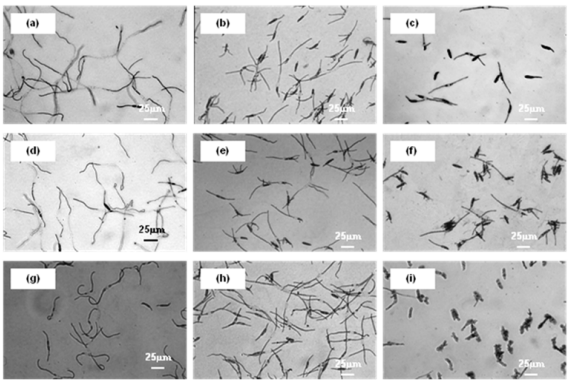

Figure 3. Germination of the basidiospore of *Exobasidium vexans* on 11 media after 19.5 hr of incubation at 22°C. (a) on Bio+Cz, (b) on Bio+PDA, (c) on Difco PDA, (d) on Pan+Cz, (e) on Pan+PDA, (f) on the home-made PSA+YE, (g) Thiam +Cz, (h)Thiam+PDA, (i) MEA.

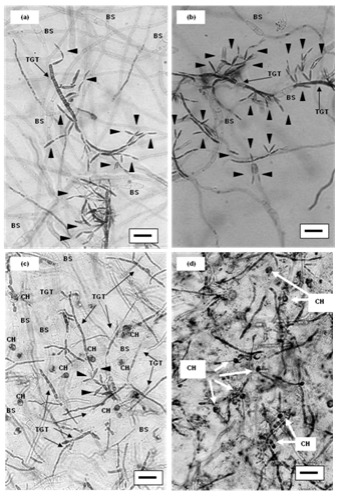

Figure 4. Germination of the basidiospore of *Exobasidium vexans* on 11 media after 96 hr incubation at 22°C. (a) on Pan+Cz, (b) on Bio+Cz, (c) on Thiam+Cz, (d) Sudan Black B staining on Pan+Cz. BS = Basidiospore, CH = Chlamydospore-like bodies and TGT = Thick germ tube. Bar = 10 µm.

B stained the chlamydospore-like bodies and condensed hyphae in black (Figure 4(d)). However, some chlamydospore-like bodies and conidia were not stained in black. At 7 days incubation, the germlings were turned to chlamydospore-like bodies and autolysed. Some germlings stayed in the condensed hyphae. After 17 days incubation, there was no visual colony but rather a mass of chlamydospores-like bodies, thick germ tubes, and condensed hyphae accompanied with or without conidia (Figure 5).

3.2 Colony induction

Colonies arose from the transferred hyphal mass including chlamydospore-like bodies and/or thick germ tubes (hereafter referred as C-bodies) on Pan+PDA and Pan+Cz. C-bodies were initiated to grow after 6 days incubation but did not develop from the inoculated agar surface. After 9 days incubation, hyphae obviously grew on the new media. Colony growth on Pan+PDA was superior to that on Pan+Cz. Colony diameter averaged 2861 μm on Pan+PDA at 27 days incubation (Figure 6). No visible colony formation occurred on the other media.

Colonies arose from the transferred thick germ tubes (hereafter referred as H-bodies) on Pan+PDA, Pan+Cz, and Thiam+Cz. However, colony induction on Thiam+Cz was recorded only once. Colony formation from H-bodies was initiated after 9 days incubation. Colony growth on Pan+ PDA was also superior to that on Pan+Cz. Colony diameter from H bodies on Pan+PDA was smaller than that which originated from C-bodies (Figure 6). Colony growth on Pan+Cz and on Pan+PDA instead of different origin of inocula was not significantly different (P = 0.05) by t-test.

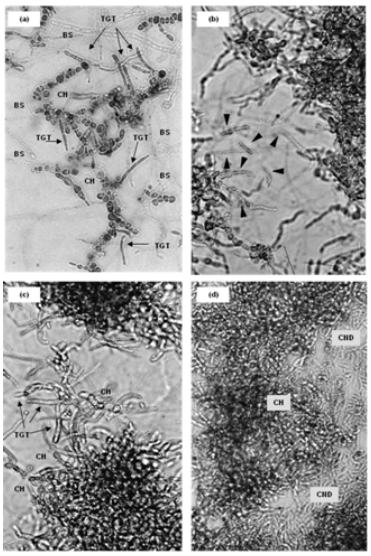

Figure 5. Morphology of growth of *Exobasidium vexans* after 17 day incubation on the media at 22°C. (a) on Bio+PDA, (b) - (d) on Pan+ PDA. BS = Basidiospore, CND = Condensed hyphae, CH = Chlamydospore-like bodies and TGT = Thick germ tube. Bar = 10μm.

Colony color of *E. vexans* was salmon (41) to flesh (37) and the colonies were composed of several components including enlarged hyphae, chlamydospores-like bodies, basidia and basidiospores (Figure 7).

Even though *E. vexans* formed small colony from basidiospores on Pan+PDA, a piece of the colony did not

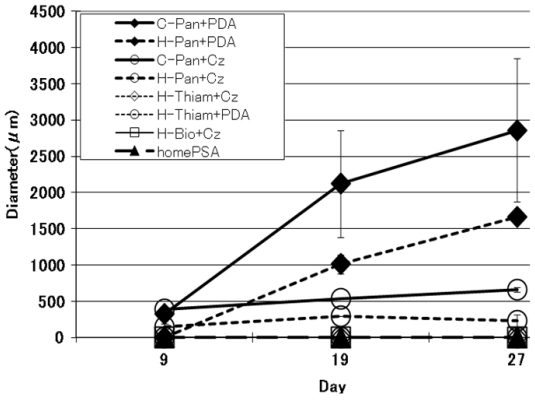

Figure 6. Comparison of colony growth of *Exobasidium vexans* after 27 days of transfer onto new media at 22°C. Error bars indicated the standard deviation. Colony growth on Pan+Cz and on Pan+PDA instead of different origin of inocula was not significantly different (P=0.05) by t-test.

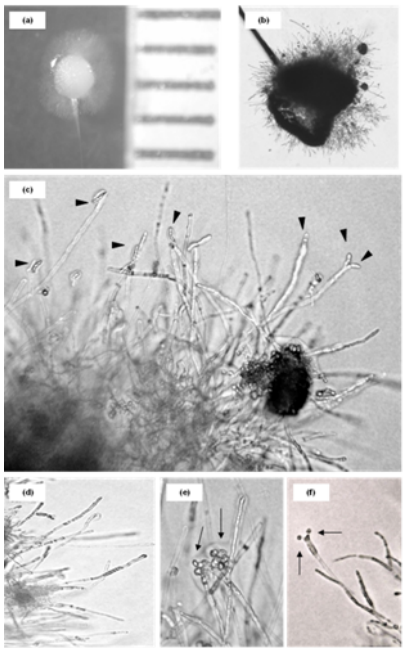

Figure 7. Morphology of colony of *Exobasidium vexans* after transferring onto Pan+ PDA at 27 day of incubation at 22°C. (a) Colony growth on the surface of Pan+ PDA. (b) Colony growth from the bottom of Petri dish. Marginal hyphae going out from agar inoculum (A). (c) Basidiospore formation on the long basidia. Arrow head shows a basidiospore. (d) Marginal hyphae. (e) Mass of basidiospore formed on basidia. (f) Initial of basidiospore on the long basidium. Bar = 1mm (a), 500μm (b) and 10μm (c-f).

grow again on Pan+PDA or Pan+Cz. No colony grew on other media examined.

Isolates H29157, H29160, H29162, and H29164 from *C. sinensis* var. *assamica* did not grow on Bio+ PDA, Pan+ PDA, and Thiam+ PDA.

4. Discussion

The *in vitro* cultivation of *E. vaccinii* (Fuck.) Woronin was first investigated by Brefeld (1889 in Graafland, 1953) in liquid medium and by Marchinatto (1929 in Ezuka, 1955) on agar medium. While success in culturing of *E. vexans* on togé-agar was reported (Reitsma and van Emden, 1949 in Graafland, 1953), *Exobasidium vexans* was presumed to be an obligate parasite (Loos, 1951 in Graafland, 1953), because of the difficulty of culturing *E. vexans*, which was still recognized as "an obligate biotroph" (Ajay and Baby, 2010; Chakraborty and Sharma, 2007). In our preliminary experiments, basidiospores naturally dispersed from the blister lesion on the media. Germination of basidiospores on the media was not a reproducible experiment. Basidiospores sometimes germinated with germ tubes but another time they did not germinate on PDA and PSA regardless to whether they were home-made or not. Germinated basidiospores stopped growth after 48 hr incubation. Naturally discharged basidiospores never germinated on MEA.

Graafland (1953) and Ezuka (1955) independently reported the successful culture of *E. vexans* on media prepared from plants (orchids and potato). Sundström (1964) used the synthetic media to cultivate *E. vexans*. From our experiments, sudden stop or no initiation of germination may be attributed to a kind of a nutrient deficiency. Colony growth of *E. vexans* was achieved on Difco PDA or Czapeck's medium amending calcium pantothenate. Thiamine was not effective on colony formation, whereas *E. vexans* was known as a thiamine heterotroph (Sundström, 1964). In this experiment, culture of *E. vexans* from basidiospores was established on Pan+PDA (Figure 6) but these cultures stayed in a dormant condition on this medium. We continue seeking the key agent to develop colony of *E. vexans* in the media prepared from plants (orchids and potato) according to the researches by Graafland (1953) and Ezuka (1955).

Ezuka (1955) recorded the course of germination and recognized three types of behavior; 1) germinated by normal germ tubes, often forming a chlamydospores-like body at each apex, 2) germinated by abnormally thick germ tubes, and secondary spores formed on them increased in number by budding saprophytically, while the older parts of these germ tubes and secondary spores gradually transformed into chlamydospores-like bodies, 3) no or only slight germination occurred and the basidiospores transformed into chlamydospores-like bodies. He reported that the second type of germination was predominant and developed to yeast-like colony. We also observed these three types of behavior (Figure 4). In addition to these behaviors, autolysis occurred on germinated hyphae independently from the chlamydospore-like structure formation in our observation.

Ezuka (1955) reported the successful culture on artificial media and the maintenance of the cultures on home-made PSA (originally described as 'potato agar' in his paper). In his report, it took more than 20 days incubation to obtain the colony growth on the home-made PSA. In our experiment, at 17 days incubation, there were no visual colonies as similarly reported in Graafland (Figure 2; 1953) and Sundström (Plate 2 (m); 1964). However, when hyphal mass (C-bodies) was transferred on new Pan+PDA or new Pan+Cz, colonies were 2861 ìm in diameter after 27 days incubation. It took more than 20 days to form visible colonies (Figure 6 (a)(b)). This result supports the observation by Ezuka (1955).

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