Penetration and Infection of Leaves of Black Walnut by *Marssonina juglandis* and Resulting Lesion Development

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ABSTRACT

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Penetration and infection of black walnut (*Juglans nigra*) leaves by the walnut anthracnose fungus and the resulting lesion development were observed. A conidial suspension was atomized onto mature leaves. Conidia germinated terminally or subterminally within 48 hr and formed short germ tubes and/or appressoria. Penetration through epidermal cells was initiated from appressoria and was either direct or followed subcuticular growth. Papilla formation was associated with resistance to penetration. Intercellular hyphae were observed after 72 hr, while intracellular spread through epidermal cells was prominent at 144 hr. By 168 hr, the mycelium had entered the mesophyll, and host cell necrosis could be seen microscopically. Macroscopic lesions were observed at 240 hr and acervulus formation occurred after 240 hr.

Additional key words: *Gnomonia leptostyla*, walnut anthracnose.

Black walnut (*Juglans nigra* L.) has become widely planted in response to an increase in the value of its timber and nuts. Many plantations are essentially monocultures and present an ideal situation for epidemic spread of foliar diseases.

Anthracnose, caused by *Gnomonia leptostyla* (Fr.) Ces. & de Not. (imperfect state: *Marssonina juglandis* (Lib.) Magn.), is the most serious foliar disease of black walnut. A five-state survey in 1975 indicated this to be the principal leaf spot disease (11). On leaflets, the necrotic lesions are ≤5 mm in diameter with small chlorotic halos. Smaller necrotic flecks (≤2 mm) appear on nut husks (5). The disease may quickly spread during wet seasons and result in premature defoliation, reduced tree growth, weakened trees, ambered nut meats, and occasional plant mortality (2,3).

Recommendations for disease control include the use of fungicides and a combination of cultural methods that promote air circulation and hasten evaporation of free moisture. Recently, applications of nitrogen fertilizers have also showed promise in reducing disease severity in tree plantations (10). At present, resistant cultivars are not commercially available. Environmental requirements necessary for disease development have been reviewed (4,5).

Details concerning conidial germination, penetration, infection, and disease development are reported here.

MATERIALS AND METHODS

The walnut anthracnose fungus was grown in petri plates on oatmeal agar (20 g of instant oatmeal, 5 g of dextrose, 20 g of agar, and 1 L of distilled water) for 2–3 wk at 21 C under a 12-hr photoperiod. Conidia were washed from the plates, collected by centrifugation, and the concentration was adjusted to 10^6 conidia per milliliter.

Seedlings of *Juglans nigra* were grown under greenhouse conditions in metal cans containing a mixture of peat:sand (3:1, v/v) with micronutrients added (5). A conidial suspension was atomized onto mature, fully expanded leaves that were then covered with plastic bags fastened around the petiole and kept for 48 hr at 21 ± 2 C. Following the removal of the bags, trees were maintained in the greenhouse with temperatures ranging from 20 to 24 C. At 24-hr intervals through 240 hr, 5-mm-diameter leaf disks were collected and prepared for examination. Beyond 240 hr, samples were collected at random time intervals.

Tissue samples were cleared in ethanol:acetic acid (1:1, v/v) for 24 hr and placed in lactophenol until translucent. Disks were stained with cotton blue in lactophenol and observed microscopically.

Additional tissue samples were fixed in formalin-acetic acid-alcohol (FAA) for 24 hr, dehydrated in a graded tertiary-butyl-alcohol series, embedded in paraffin, and sectioned. Sections were stained with cotton blue in lactophenol.

Observations were also made by scanning electron microscopy. Nine-millimeter-diameter leaf disks were collected from inoculated trees, fixed in 2% osmium tetroxide at 4 C for 24 hr, dehydrated in a graded ethanol series, dried in a critical-point drier, mounted, and coated with gold.

RESULTS

Observations 48 hr after inoculation. From 60 leaf disk samples and approximately 5,000 conidia observed per leaf surface, less than 1% of the conidia inoculated onto the lower surface of leaves had formed germ tubes in contrast to 14% inoculated onto the upper surface. Conidia produced from one to three germ tubes 1–3 times the length of the conidium at terminal or subterminal positions (Figs. 1 and 2). Scanning electron micrographs showed conidia with a gelatinous substance that appeared to cement them to the host surface (Fig. 2). Occasionally the gelatinous material seemed to form a bond between conidia. Germ tubes were never observed to form distinct appressoria. Appressoria were observed within 48 hr either at the terminal end of a conidium or subterminally, where contact was made with the host surface (Figs. 3 and 4).

Observations 72–96 hr after inoculation. Penetration was accomplished via a penetration peg from an appressorium either directly into an epidermal cell or after a period of subcuticular development (Fig. 4). In veinal epidermis, septate, subcuticular hyphae commonly were formed, causing the cuticle to bulge. These hyphae grew to 2–3 times the length of a conidium and parallel to the long axis of the host epidermal cell (Fig. 5). Later, a hyphal branch growing perpendicularly from the subcuticular hyphae penetrated the cell wall and branched repeatedly as it entered the cell lumen. Subcuticular hyphae were not observed in interveinal epidermal cells.

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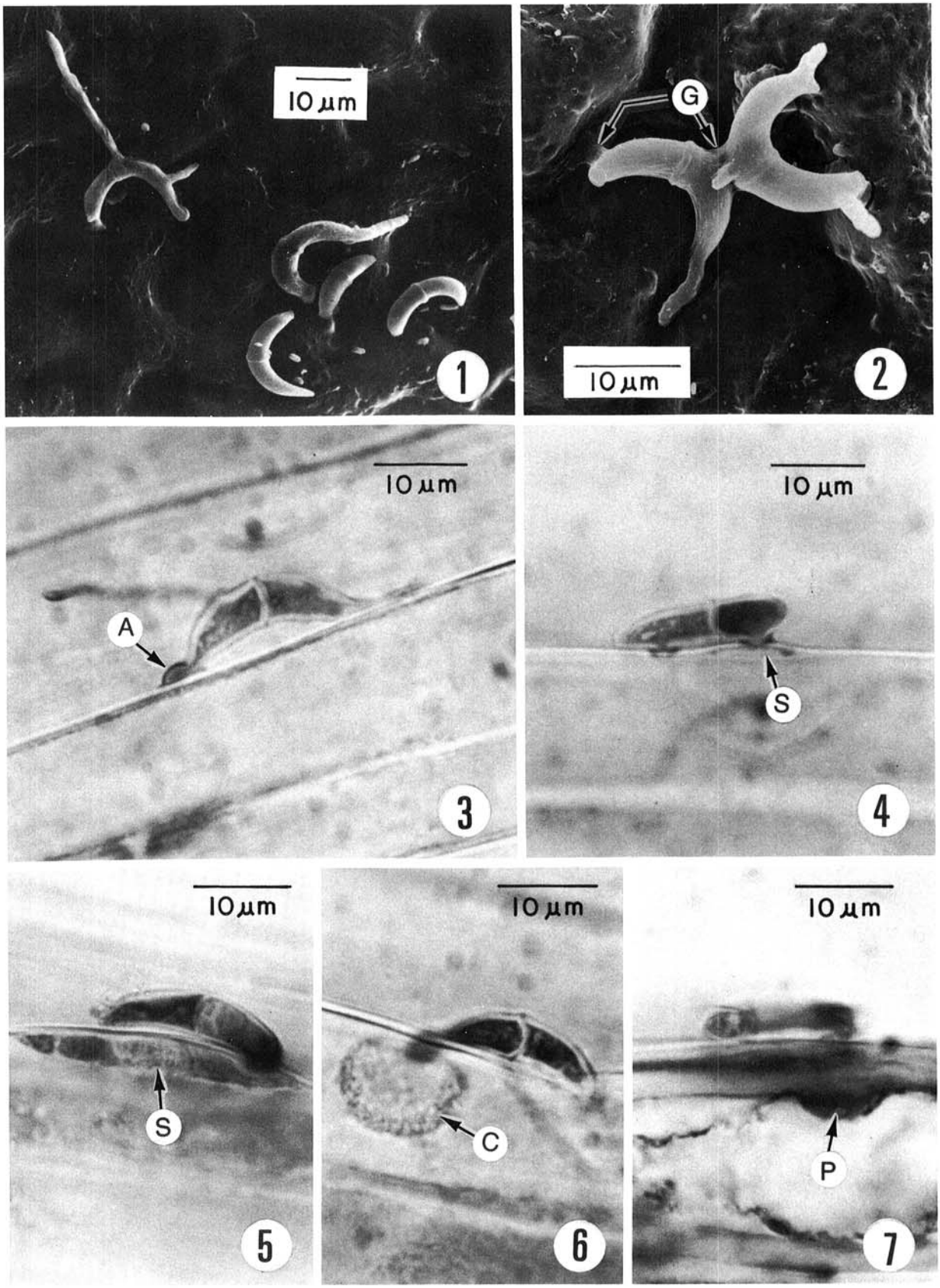
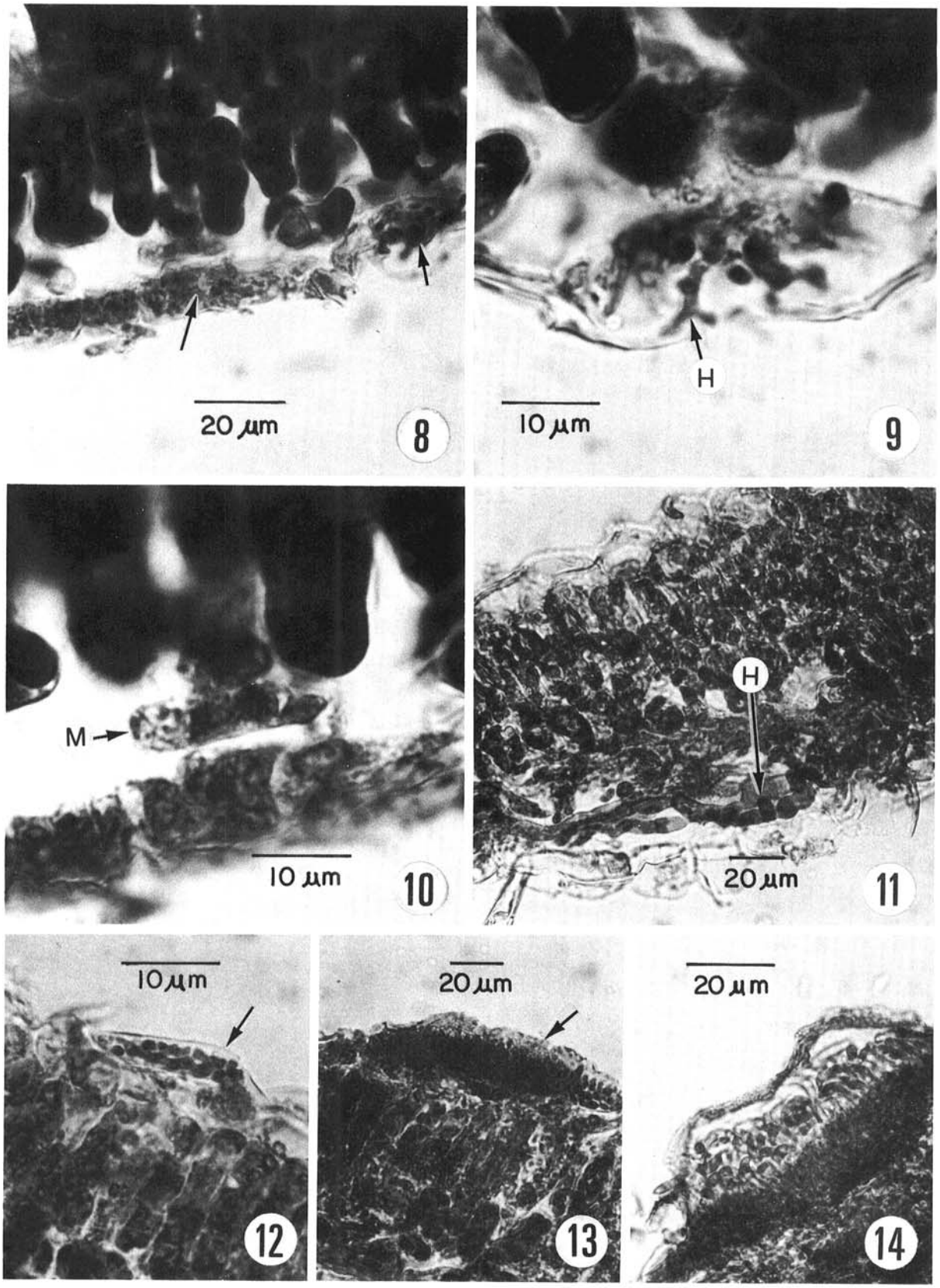


Figs. 1-7. Photomicrographs of the initial stages of germination, penetration, and infection of black walnut by *Marssonina juglandis*. **1,** Scanning electron micrograph showing conidia with terminal and subterminal germ tube development. **2,** Gelatinous material (G) between conidia and on the epidermal surface. **3,** Conidium displaying a terminal appressorium (A). **4,** Conidium with two subterminal appressoria and an initial stage of subcuticular growth (S). **5,** A septate, subcuticular hypha (S) produced beneath a conidium, causing the cuticle to bulge. **6,** Accumulation of a granular cytoplasmic aggregate (C) in an epidermal cell below an attempted penetration site. **7,** Cross section showing a papilla (P) formed beneath a conidium on the epidermal cell surface.



Figs. 8-14. Developmental stages of *Marssonina juglandis* infection from the colonization of black walnut leaf tissue through acervulus formation. **8,** Lateral spread of hyphae through the lower epidermis (arrows). **9,** An epidermal cell showing intracellular hyphae (H). **10,** Higher magnification of Fig. 8 showing the granular appearance of a mesophyll cell (M), indicating colonization. **11,** Intercellular hyphae (H) ramifying throughout the mesophyll region. **12,** Initial stage of acervulus formation; hyphae proliferating within the upper epidermis (arrow). **13,** Further acervulus development, showing hyphal multiplication causing the cuticle to bulge (arrow). **14,** Conidiogenesis within the developing acervulus; intact cuticle.

On occasion, attempted penetrations of veinal epidermal cells resulted in the accumulation of a granular cytoplasmic aggregate below the attempted penetration site (Fig. 6). From paraffin sections, papillaelike structures (12) were observed in the same region as cytoplasmic aggregation (Fig. 7). When papillae were formed, further penetration ceased. The presence of a refractive halo surrounding the penetration site provided further evidence for papilla formation in interveinal epidermal cells. In general, papilla formation occurred infrequently in all tissues.

Individual cells were substantially colonized by 96 hr and contained extensive intracellular hyphae. As cells became infected, the contents appeared granular and stained deeply, which indicated the presence of the fungus.

Observations 120–168 hr after inoculation. By 120 hr, intracellular hyphae were observed to have spread to adjacent veinal epidermal cells through plasmodesmata. By 144 hr, hyphae branched and eventually filled the intracellular space. Lateral spread in the interveinal epidermis preceded colonization of the mesophyll (Fig. 8). At distal regions from the initial penetration site, distinct intracellular hyphae could be seen in epidermal cells (Fig. 9). As the infection progressed, the cytoplasm of spongy parenchyma cells appeared to become granular (Fig. 10). Subsequently, hyphae were observed in the mesophyll intercellular spaces (Fig. 11). By 168 hr, microscopic cell necrosis could be seen. Palisade cells became irregular, lost turgidity, and collapsed.

Observations >240 hr after inoculation. Cellular integrity was destroyed by the proliferation of hyphae, and in certain epidermal regions acervulus formation was initiated (Fig. 12). Further hyphal growth forced the cuticle to bulge outward (Fig. 13). Eventually, short conidiophores produced the characteristic two-celled conidia, forcing the rupture of the cuticle and the liberation of spores (Fig. 14).

DISCUSSION

The development of walnut cultivars resistant to anthracnose has not been actively pursued. A preliminary study (4) suggested that a large variation in resistance among hybrids of *Juglans* species does exist. Information concerning the establishment and development of *M. juglandis* in black walnut leaves may facilitate a direct approach to a disease resistance screening program for black walnut and aid in the interpretation of the success or failure of other control measures. In addition, intensification of the disease to epidemic levels is a function of the conidial production during multiple infection cycles. Previous studies have not examined aspects of conidial germination, penetration, and lesion development in the host-parasite association.

Matteoni (8) showed that abaxial surface inoculations resulted in a 10-fold increase in lesion development as compared with adaxial surface inoculations. He suggested the possibility of conidial infection through stomata or trichomes. Neither the penetration of trichomes nor germ tube attraction toward stomata was observed in this study.

Conidia were found to form germ tubes more frequently when inoculated onto the adaxial surface, while appressoria were never formed in association with germ tubes. Conversely, germ tubes were rarely associated with conidia inoculated onto the abaxial surface, while directly produced terminal or subterminal appressoria were common. Aronescu (1), studying Marssonina black spot of rose, observed that long germ tubes were seldom found, and if produced, they failed to make appressoria. Contact and chemical stimuli have been used to explain differences in germ tube and appressorial formation with various fungi (7,9). Mercer (9) noted a suppressive or stimulative effect on appressorium formation with respect to the leaf surface for *Colletotrichum lindemuthianum* and suggested that nutrients were responsible for this effect. Similar results were shown by Grover (7) for *Colletotrichum piperatum* on pepper; he concluded that conidial germination and appressorium formation, although chemical dependent, were independent processes.

The gelatinous substance secreted around conidia appears to be of fungal origin in the form of a secretion. Only conidia that germinated or produced appressoria were coated with this substance. Others have observed similar gelatinous materials associated with anthracnose fungi (1,13).

Subcuticular hyphae were associated with veinal epidermal cells but not with interveinal epidermal cells. Anthracnose fungi commonly produce subcuticular hyphae (1,13). These hyphae have been associated with the latent stage in immature tissues and are thought to be produced in response to cellular toxins or to the composition of the cell wall at differing periods of host maturity (6,13). The cell wall of veinal epidermal cells is considerably thicker than other leaf epidermal cell walls. While this does not present an impenetrable barrier to the anthracnose fungus, the common occurrence of subcuticular hyphae may reflect a mechanical barrier to immediate penetration.

Attempted penetrations sometimes resulted in a host-cell reaction that appeared to be equivalent to papilla formation. These structures formed in veinal epidermal cells and were less frequently observed in other epidermal cells. Granular zones were produced immediately underneath conidia and below the host cell wall within 72 hr after inoculation. Cross sections revealed these to be inward-projecting extensions of the cell wall and to resemble papillae formed in other plants inoculated with various pathogenic and nonpathogenic fungi (12). Where papillae formed no further penetration was observed. Thus, papillae appeared to impart some resistance to disease development; however, this mechanism of resistance is thought to be of minor importance.

Acervulus formation typically begins from 10 to 14 days following inoculation at temperatures around 21 C. This period is extended when temperatures increase (8). Matteoni (8) observed that acervulus production was associated with leaf age. Juvenile leaves developed smaller lesions and produced either fewer or no acervuli. Similar observations on the formation of acervuli in immature fruit have been reported (6,13). The relation between plant juvenility and fungal reproduction in black walnut is not understood but may have some importance in the development of anthracnose epidemics. This form of disease resistance is currently being investigated.

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