A NEW MUCOR-LIKE FUNGUS FROM PLANT ROOTS

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In 1941, the writer obtained in his laboratory, as a chance isolation from roots of a diseased American elm growing in Peoria, a Mucor-like fungus that was producing both sporangia and zygospores in great abundance. The method of zygospore production revealed that the mold belonged to *Zygorrhynchus*, a genus in which, in contrast with Mucor and Rhizopus, zygospores result from the union of heterogametes formed by a homothallic mycelium. Since no species of this genus has thus far been recorded in Illinois, this account serves first to record this kind of fungus in the State. Also, it describes the Illinois isolation as the type of a new species and offers some observations on the process of zygospore formation.

TAXONOMY

In technical literature six species of *Zygorrhynchus* have been named and described. Five of these are European and one is Japanese. For the most part they have been segregated as species on seemingly trivial characteristics, just as have the species of Mucor and Rhizopus, and the descriptions of them are incomplete.

In America, *Zygorrhynchus* has been isolated a number of times from soils. According to Henrici (1930, pp. 47, 70), because it is active in ammonification, it is one of the most important soil-inhabiting Phycomycetes. With the exception of the isolation made by Povah (1917) in Michigan, which he determined as *Z. Vuillemini* Namysl., American isolations have been classified as *Z. Moelleri* Vuill. Unfortunately, *Z. Moelleri* is the most poorly described of the named species. Vuillemin (1903) distinguished it, as the second species of the genus, almost wholly on certain differences apparent to him between its spores and zygospores and those of *Z. heterogamus* Vuill., the one previously described species. Saccardo[1] may have added confusion by stating the zygospore difference in reverse in the Sylloge Fungorum. Judging, however, from such accounts as that of Grossman (1911), it seems highly improbable that American isolations are the *Z. Moelleri* of Vuillemin. The Illinois isolation certainly is not that species and appears sufficiently distinct from all described species to deserve recognition as another species.

Zygorrhynchus verruculosus n. sp.[2]—Mycelium hyaline (gray in mass), coenocytic, intricately branched but scant and confined to the substratum; main hyphae in age becoming yellowish, 14-16 μ in diameter, densely verruculose, in vigorous growth 8-12 μ in diameter, often verruculose; branches progressively finer and smooth. Chlamydospores abundant, terminal and intercalary, solitary or in short chains, elliptical, oval or subspherical, 13 × 7 μ to 30 × 20 μ. Sporangia abundant, spherical, black, irridescent, 45-55 μ in diameter, apical on hyaline, usually simple but often bifurcate, rarely less regularly branched, densely verruculose sporangiophores 8-10 μ in diameter and 100-300 μ long (when grown in the dark, rare and on sporangiophores many times longer); columella small, spherical to applanate, hyaline, smooth, 15-20 μ in diameter; tunic densely studded with very fine, raphidic spicules, disintegrating completely into innumerable irridescent red, purple and blue particles, leaving no collar. Spores hyaline, oblong-

[1]"Z. Moelleri Vuill.—Bul. Soc. Myc. 1903—A *Z. heterogamo* differt sporis ellipsoideis, 4.3 × 2.6 μ, nec globosis zygosporisque duplo triplove majoribus."

[2]Zygorrhynchus verruculosus n. sp.—Mycelio hyalino, 8–12 μ vel in aetate lutescente, 14–16 μ diametro, dense verruculoso. Chlamydosporis terminalibus et intercalaribus, 13 × 7 μ ad 30 × 20 μ. Sporgangiis sphaericis, nigris, inridescentibus, 45–55 μ diametro, in sporangiophoris simplicibus vel bifurcatis, 8–10 μ diametro, 100–300 μ longis, dense verruculosis latis; collumella sphaerica vel applanata, 15–20 μ diametro; tunica minute spiculosa, totaliter disrumpente et collarem non relinquente. Sporis hyalinis, continuis, binucleatis, oblongo-ovalibus et allantoideis, 4.5–5.1 × 2.9–3 μ. Hyphis zygophoris aereis, hyalinis, 5–8 μ diametro, dense verruculosis. Zygosporis globosis, nigris, 45–60 μ, maximo numero 50 μ diametro, denticulis pyramidalibus 6–8 μ altis dense ornatis et cum tympano majore circiter 25 μ diametro porum 1.5–2 μ diametro possidente. Azygosporis nullis.

ovate and noticeably allantoid, binucleate, nonseptate, 4.4-5.1 × 2.9-3 μ. Zygophoric branches aerial, hyaline, 5-8 μ in diameter, densely verruculose. Zygospores globose, black, 45-60 μ, the majority about 50 μ, in diameter at maturity, densely studded with pyramidal denticles 6-8 μ tall, 5-6 μ wide and irregularly stellate at the base; major tympanum about 25 μ in diameter and provided (apparently) with a central pore 1.5-2 μ in diameter. Azygospores not produced.

Obtained from rotting roots (possibly from adhering soil) of *Ulmus americana* L. from Peoria, Illinois, in 1941. Specimen and slides in the Illinois Natural History Survey herbarium, accession number 28927 (TYPE).

In addition to the characters required for technical description, the Illinois isolation displays others, less definite, which seem to set it off from the other species. One of these is its reaction to light in the production of sporangia and zygospores. It has been said of the majority of the earlier species that sporangia occur sparingly. With the Illinois isolation sporangia occur abundantly when cultures are grown in light, a new colony producing hundreds of them in mature condition within three or four days. If, however, cultures are grown in darkness, only a few sporangia develop during a period of many days; then, also, they occur on very long and almost always unbranched sporangiophores. Zygospores, on the other hand, develop slowly in cultures exposed to light. With Van Tiegham cell cultures set up for observation and maintained in a light room, four to six days may pass before zygospores begin to form in numbers, and early stages of zygospore formation exposed to concentrated light while being observed under the microscope are often arrested in development and afterwards make no further progress. Observational microcultures make little or no progress toward zygospore production during daylight hours but produce large numbers of zygospores during the night. On the other hand, new microcultures kept in a dark culture chamber produce zygospores in hundreds and in all stages of development in 36 to 48 hours.

A somewhat distinctive characteristic is seen also in the aberrancies of gametic fusion. In this species, as in others, contact and fusion normally occurs between closely related branches (fig. 15). Nevertheless, a great amount of successful contact occurs between distantly related branches: minus gametophores make contact, and successfully produce zygospores, with plus gametophores arising not only from different aerial branches but even from distinctly separate vegetative hyphae. With this ability is associated an exceptional length of the minus gametophore (as indicated, at least, by the figures of other authors). Also, complete reproductive capacity is often retained in the plus gametophore. This structure not infrequently becomes septate and gives rise to one or more branches which, in turn, produce both plus and minus gametophores and, eventually, normal zygospores.

In the descriptions, or in discussions, of the previously described species there usually is some mention of the production of azygospores. During the two years the Illinois isolation has been under observation, no azygospore has been found in any of the cultures. In this respect, the Illinois isolation differs not only from most of the previously described species but also from some American isolations (Blakeslee, 1904).

The position of the Illinois isolation as a species among the species already described is indicated with some accuracy by the following key, in which the principal distinctions noted by the describers of the other species are given prominence.

Key to the Species of Zygorrhynchus

Spores globose, 2.2-2.7 μ in diameter; zygospores usually more than 100 μ in diameter . . . . . *Z. heterogamus* Vuill.

Spores elliptic, oval or oblong; zygospores much smaller.

- Spore elliptical, uniformly small (about 4 μ long) or variable and larger.
  - Columella pyriform; spores variable (6-10 × 1.5-3 μ); zygospores 68 to 80 μ in diameter . . . . *Z. japonicus* Komin.
  - Columella applanate spherical; spores small.
    - Zygospores small, usually about 35 μ in diameter; spores 4.3 × 2.6 μ . . . . *Z. Moelleri* Vuill.

Zygospores larger, 40-45 μ or up to 60 μ in diameter; spores 4 × 2 μ. . . . . *Z. Vuillemini* Namysl.

Spore oval or oblong, small.

Zygosporic denticles usually caespitose. . . . . *Z. Bernardi* Moreau

Zygosporic denticles not caespitose.

Zygospores small, 14-48 μ in diameter; denticles 3-5 μ long, often recurved. . . . . *Z. Dangeardi* Moreau

Zygospores larger, 45-60 μ in diameter; denticles 6-8 μ long, pyramidal and not recurved . . . . *Z. verruculosus* Teh.

A point not given attention in studies made of earlier species is the nuclear condition of the spores. The spores of *Z. Vuillemini* were described as guttulate, those of *Z. Dangeardi* as 1-2 guttulate; but guttules are not characteristically identifiable as nuclei. The spores produced by the Illinois isolation are obviously and clearly binucleate. This fact may indicate that plus and minus nuclei are carried together, but as separate entities, in the coenocytic mycelium from the germination of the zygospore of one generation to the formation of the zygospore of the next generation, even though many reproductions by spores intervene.

Germination of mature zygospores of the Illinois isolation has not been obserbed. Often, in culture transfers, an apparent germination resembling that reported by Green (1927) has been seen, but it is very doubtful whether this was true germination. Since sporangia are produced freely and are closely intermixed with the zygospores above the surface of the colony, it is possible that spores lodged among the denticles ornamenting the zygospores and, transferred with the zygospores, germinated without being dislodged when presented with fresh medium.

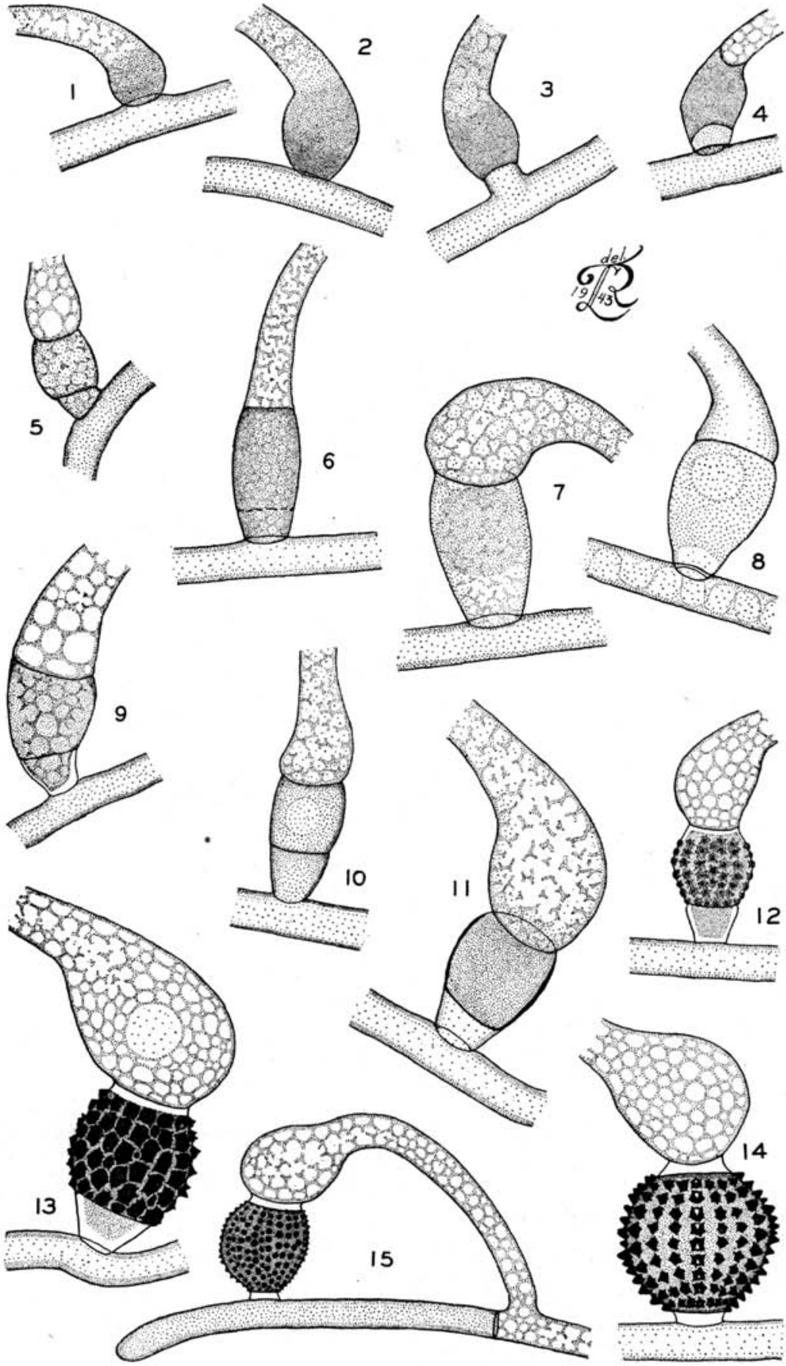
Process of Zygospore Formation

The production of zygospores by species of Zygorrhynchus has received a considerable amount of study, and three views have been expressed by observers, all of whom agree on the major features of the process. These features, described by Vuillemin (1903) and Blakeslee (1904) and later by others, are as follows: From the vegetative mycelium, aerial hyphae arise. These aerial hyphae give rise to lateral branches upon which the zygospores are produced. A lateral branch first becomes divided at some point by a septum that differentiates it into a basal and a distal portion. A branch then arises from the basal portion, just below the newly formed septum, and grows forward, curving back until its tip makes contact, beyond the septum, with the side of the distal portion of the branch from which it arose. At the point of contact, a series of changes takes place and results in the zygospore (fig. 15). It is with regard to this series of changes that investigators have disagreed.

According to Vuillemin (1903), when contact between two zygophoric branches has been made, the distal portion of the original branch produces at the point of contact a short lateral branch which remains apically in contact with the apex of the touching branch. Within this short lateral branch and, simultaneously, within the touching branch walls are laid down, cutting off cells that function as gametes. The gamete cell in the short lateral branch is small while that in the contacting branch is large. Following formation of the gametes, the wall dividing them disappears and, from the two, the zygospore develops. This process has been substantiated in the observations Blakeslee (1904, 1913) made, but appears to have been modified to some extent by the observations of Green (1927), whose account and small inadequate drawings leave some doubt as to how clearly all of the stages, especially the fusion of the gametes, were observed.

Atkinson (1912) at first professed to have found no male gamete. He thought that no male progametangium arose and that the tip of the touching branch fused with the distal part of the original branch, that dissolution of the separating wall occurred, and that direct transfer of protoplasm and nuclei from minus to plus zygophore took place. Later (Blakeslee, 1913) he retracted this view. Grossman (1911), describing an isolation he made in Michigan, expressed essentially the same view, but it may be doubted whether he intended his very brief exposition to be taken literally.

Gruber (1912), expressing a third opinion, believed that, after contact had



Figs. 1–15.—*Zygorrhynchus verruculosus,* n. sp. Stages in conjugation and development of the zygospore. (See p. 113 for explanation of figures.)

been made by the two branches, a branch grew out from the side of the touched hypha, that this branch elongated, that it became divided by a cross wall, and that its terminal cell became swollen and was fertilized by the passage into it of protoplasm and nuclei from the now-swollen end of the touching hypha. According to his view, the minus gametangiophore of Vuillemin and Blakeslee would be the plus gametangeophore and the plus gametangeophore of Vuillemin and Blakeslee would be the minus gametangeophore. Moreau (1912) immediately took exception and claimed that Gruber's interpretation was erroneous.

Of these three interpretations, that of Vuillemin and Blakeslee is generally accepted, owing to Blakeslee's recognized authoritativeness regarding fungi of the Mucorales, to Moreau's denial of Gruber's conclusions, to Atkinson's retraction of his view, and to the later substantiation of Vuillemin's observations by Blakeslee (1913) and by Green (1927). Actual observation of some of the stages is, however, made extremely difficult by both the small size of the cells observed and the obscurity imposed by the early development of cell wall ornamentations. It is not surprising, therefore, that more than one interpretation has been made or that some of the stages have not been clearly observed.[3]

The stages of zygospore formation exhibited by the Illinois isolation have been followed carefully with both actively growing and killed and stained material. To observe growing material, the method used by Blakeslee (1913) was modified by the substitution of Van Tieghem cells for watch glasses and glass covers. A small drop of agar medium, spread thinly while hot over a part of the surface of a coverglass, was inoculated with a small bit of the mold, and the coverglass was then inverted and sealed to the top of the Van Tieghem ring. With a supply of sterile water in the base of the Van Tieghem cell, such a microculture developed well and, especially at the periphery of the coverglass where no medium interfered with light, examples of development could be selected and watched with the ordinary high (44 × or 4 mm.) and sometimes with the oil (97 × or 1.8 mm.) objectives. Since the hyphae on which zygophoric branches occur are almost always aerial, the chief difficulty lay in finding examples suitably oriented for study. Both Blakslee (1913) and Green (1927) have given time schedules with their illustrations of progressive stages; this it has been impossible to do with the present fungus because of its sensitiveness to light.

To obtain material for staining, microcultures were made in small drops of nutrient broth on clean slides. The cultures were placed in inverted sterile Petridishes and the gaps between covers and bottoms of the dishes were sealed with sterile water to maintain a saturated atmosphere in the dishes. These cultures were grown in an incubation chamber into which no light entered. After 36 to 48 hours they were removed and treated for 24 hours with standard formalin-aceto-alcohol solution as a killing agent. Safranin for 24 hours, followed in order by acidified alcohol, ammoniated alcohol, and analine blue in alcohol for two mniutes, proved most satisfactory as a gross structure stain, giving beautifully clear differentiation of chlamydospores, mature sporangiospores, and the swollen zygosporic suspensors. The method would be suitable for the preparation of slides as permanent teaching equipment.

In the Illinois isolation the hyphal apparatus involved in zygospore production is essentially the same as that described for other species. From any point on a vegetative hypha a specialized aerial branch may arise. From this aerial

[3]Regarding actual conjugation, Blakeslee (1913) wrote, "There seems no doubt also that these two gametes . . . unite later by the dissolution of the intervening cross wall into a single cell. . . ."

Explanation of Figures

Fig. 1.—The tip of one zygophoric branch touches and adheres to the side of another zygophoric branch. Fig. 2.—The touching tip becomes large and bulbous. Fig. 3.—The touched branch produces a small outgrowth. Figs. 4–5.—Cross walls separate the bulbous tip and the outgrowth as gametangia. Fig. 6.—The double wall between the gametangia dissolves. Fig. 7.—The fused gametangia begin to enlarge and form the zygospore. Figs. 8–10.—A wall, possibly the endospore, appears in the lower part of the zygospore. Also, swelling of the touching branch above the zygospore becomes pronounced. Fig. 11.—The zygospore wall increases in thickness. Fig. 12.—Dark thickenings appear in the outer wall of the zygospore. Figs. 13–14.—The zygospore increases in size, its polar tympana become evident, and its wall ornaments take form. Figs 15.—The characteristic zygophoric structure, with an immature **zygospore.**

branch, in turn, lateral branches arise, increase in length for some time, and then produce at their tips two bud-like outgrowths. The two outgrowths appear almost simultaneously. One of them elongates in a forward direction and, although it remains of lesser diameter, appears as a continuation of the branch on which it arose. It is, however, of determinate length, and its contents are soon abjoined by a cross wall, formed near its base, from the contents of its parent branch. The second outgrowth elongates at first in an obliquely forward direction and appears as a lateral branch. Its contents are not, as a rule, abjoined by a cross wall from those of the parent branch. As it elongates, it arches toward, touches, and eventually adheres by its tip to the side of the branch developed from the first bud. When it touches, the zygospore forms (fig. 15).

Immediately after the second branch has touched the first, its tip begins to enlarge (fig. 1). Enlargement continues until a bulbous structure (fig. 2) is formed, in which a dense concentration of protoplasm takes place. At the point of contact between the two branches, the touched branch now produces a short lateral outgrowth (fig. 3) which, as it develops, pushes the touching branch tip backward. This outgrowth, often only 1.5 μ long and rarely as much as 3 or 3.5 μ long, is so short that it can hardly be called a branch. While it is taking form, a cross wall develops in the touching branch in such a position as to separate its bulbously enlarged end as a gametangium. Almost simultaneously a cross wall also develops in the short outgrowth from the touched hypha, usually at or near the level at which the outgrowth originated, to form the other gametangium (figs. 4 and 5).

When the gametangia have thus been defined, their fused walls begin to dissolve (fig. 6). At this stage there usually is not much constriction apparent in the region of the cross walls. With their dissolution, a mingling of cytoplasm and nuclei from the two gametangia occurs and enlargement of the united gametangial cells proceeds rapidly (figs. 7 and 8).

It has not been possible to determine either how much cytoplasm or how many nuclei are contributed by each gametangium. Since one gametangium is small and one is large, it may be supposed that each contributes cytoplasm in proportion to its cubic capacity. The nuclei, during this period, either are too small to be accurately observed or are obscure in the dense cytoplasm. In mature zygospores sectioned two or three months after they had attained full development eight nuclei were counted. Four of these nuclei were large, measuring about 3.5 μ in diameter, while four were small, measuring about 2.2 μ in diameter. They were scattered in the parietal layer of cytoplasm and appeared to have no regular arrangement—at least, there was no grouping together of the small or the large nuclei, no regular alternation in position of large and small individuals, and no obvious pairing.

At an early stage in the enlargement of the united gametangia an indication can be seen that a wall is to be formed in the end connecting with the touched branch (fig. 8). The growth of this wall is accompanied by absorption of protoplasm into the zygote (fig. 9). Although constriction is usually observable where this wall develops, it is questionable whether this wall connects directly with the gametangial wall. It may be the developing endospore.

At this time, or earlier, the touching hypha begins to enlarge in the region next to the zygospore (figs. 5-9) and, as the zygospore grows, continues to swell into a great bulb filled with highly vacuolated protoplasm (figs. 10-15). After union of the gametangia, the touched hypha remains turgid, but it contains little protoplasm and appears to have no function other than that of supplying some mechanical support for the zygospore. The greatly swollen part of the touching branch, which now may be called a suspensor, apparently assumes in full the function of supplying nutriment to the developing zygospore. Also, it soon becomes densely studded throughout with minute, low, nearly hyaline wart-like thickenings. The touched branch remains smooth.

The zygospore, as soon as it begins to enlarge, begins both to thicken its wall (fig. 11) and to initiate on its wall a regular series of thickenings (fig. 12) which develop into pyramidal, black teeth (figs. 13-14). On the mature zygospore (not shown in the figures), these denticules are regularly spaced and are so numerous that they completely cover the surface of the zygospore. They reach a height of 6 to 8 μ and, at their bases, are irregularly stellate.

For a long time after the zygospore matures, it remains attached to the hyphae that formed it. At the two points of attachment circular tympana, one one large, and one small, separate it from its suspending hyphae. These tympana become evident early in the zygospore's development (fig. 12), remain visible during much of the growth period (figs. 13-15), and are finally hidden when the zygospore approaches full size. Both tympana are hyaline and relatively thick (1.5 μ), and in each there is a central pit or pore through which, it may be supposed, protoplasmic contact and translocation is maintained.

The wall of the mature zygospore consists apparently of the same five layers Vuillemin (1904) destinguished. The outermost layers, two in number, form a dark, hard, denticulated exospore, and the inner layers, three in number, form a hyaline, cartilaginous, smooth endospore. Under pressure the brittle exospore readily breaks into fragments, leaving the zygote enclosed by the tough endospore.

The origin of some of these wall layers is to some extent obscure. The exposure, which includes a dark, very thin cuticular layer and an inner, thick, more or less carbonaceous, denticle-studded layer, develops directly from the original walls of the gametangia. It is continuous with, and represents the same structural unit as, the inclosing wall of the mycelium. The modification of its surface sets it apart, however, as a specialized capsule in which the zygospore is held. The endospore, which consists of a very thin outermost layer (the middle cuticular layer), a thick, elastic, cartilaginous layer, and a very thin, granular innermost layer, could possibly be formed as a series of additions to the inner surface of the exposure. If this were the case, a tight union between exospore and endospore would be expected. Since, however, the two do separate readily, the endospore could be formed by the fused protoplasts from the two gametangia as an entirely new wall serving to separate the zygote as a complete new unit. The meager evidence that has been visible in sectioned material tends to support the view that the endospore is a separately formed unit.

Summary

Occurrence of a fungus of the genus *Zygorrhynchus* in the State of Illinois is reported for the first time. Characteristics of the zygospores, sporangia, spores and mycelium distinguish it from the six described species and, as a new species, it is named *Z. verruculosus*. Its sporangiospores are binucleate and transmit the heterothallic condition through asexual generations. Zygospores are formed in the manner characteristic of the genus. At maturity, they contain eight nuclei, of which four are large and four are small, and are inclosed by an exospore and an endospore. The exospore is formed from the gametangial walls; but the endospore, it is suggested, is formed as an independent structure.

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