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Location and activity of hyphae of the downy mildew, *Peronosclerospora noblei* (Family Peronosporaceae), and its relationship to symptom expression on wild sorghum (*Sorghum leiocladum*)

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Abstract. A study of the temporal and spatial relationships between the downy mildew, *Peronosclerospora noblei* and its host, *Sorghum leiocladum* (wild sorghum) has been undertaken. Hyphae of *P. noblei* perennate in the tiller bases of wild sorghum when the grass is dormant over the winter months. In early summer, hyphae grow into some tiller buds but not into others. Tillers that develop from invaded buds become systemically invaded by the hyphae, in most cases remain vegetative and bear the anamorph and teleomorph of the downy mildew. Tillers that develop from buds that are not invaded become short vegetative tillers or tall flowering tillers. On infected tillers symptom development is delayed until at least the third leaf, as a direct result of hyphal activity in the developing tiller. The location and activity of hyphae of *P. noblei* in tiller bases and developing tillers are responsible for the perennation of the pathogen and for the expression of symptoms in infected tillers of *S. leiocladum*. There may be similar relationships between hyphal location and activity and symptom development in other systemic pathogens of perennial grasses.

Introduction

Ryley (1985) has demonstrated that the asexual and sexual states of the downy mildew, *Peronosclerospora noblei* (Weston) C.G.Shaw, are confined to chlorotic leaves of tall vegetative tillers that develop from tussocks of its only known host, wild sorghum [*Sorghum leiocladum* (Hack.) Hubb.]. This densely tussocked, caespitose perennial grass is found along the Great Dividing Range of eastern Australia between southern Queensland and northern Victoria (Hubbard 1938). Healthy tussocks are composed of short vegetative tillers (up to 30 cm high) that are present throughout the year and tall flowering tillers (up to 1.7 m high) that develop over one short period during the summer months (October–February). Downy mildew-infected tussocks have short vegetative tillers, tall, infected vegetative tillers and may or may not possess flowering tillers. Infected tillers grow over the same period as flowering tillers and both break up readily after maturation.

Although tillers infected by *P. noblei* approximate the heights of healthy flowering tillers, they have significantly more nodes [6–(7.8)–11] than the flowering tillers [2–(3.2) 4]. The lowermost two to four leaf blades on infected tillers are dark green and narrow (1.0–3.6 mm wide) and similar to those on flowering tillers. The first infected leaf blade is only partly chlorotic, but the blades of subsequently formed leaves are entirely chlorotic, wider (5.0–9.0 mm) than normal leaf blades and are held in an upright manner. The short internodes in the upper part of the infected tillers together with the upright chlorotic leaf blades give infected tillers a bunchy-top appearance.

Asexual sporulation occurs only on the abaxial surfaces of chlorotic leaf blades, starting at the distal portions and progressing towards the base. Oogonia develop in the infected leaf blades after asexual sporulation has ceased. The reproductive structures of *P. noblei* never develop on the leaf blades of short vegetative tillers or flowering tillers even when these tillers occur in the same tussocks as tall infected tillers. Individual infected tussocks produce infected tillers year after year even after they have been cut back to a short stubble at the end of the previous growing season (Ryley 1985). These observations suggest that infected tillers are systemically invaded by hyphae that perennate in the tiller bases.

The relationships between the location and activity of hyphae and the development and expression of symptoms on infected tillers have been documented for many downy mildews that infect cultivated annual grasses, e.g. *Sorghum bicolor* (L.) Moench, *Zea mays* L. and *Pennisetum americanum* (L.) K. Schum. However, little is known about these relationships for downy mildews of wild perennial grasses. This paper reports on the relationships between the spatial and temporal activity of hyphae and symptom

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development. The study involved histological examinations of host tissue from tiller bud formation through to tiller maturity.

Materials and methods

A total of 20 diseased tussocks and 20 healthy tussocks were removed from a population of wild sorghum at Hirstglen, south-eastern Queensland (27°49′06″S, 152°06′14″E) and transported to the University of Queensland for laboratory examination. The individual tiller bases, which together comprised the tussock, were carefully separated and the tillers were designated as healthy, infected or vegetative depending on the nature of the above-ground parts. Healthy tillers bore inflorescences, infected tillers had wide chlorotic leaves and/or frayed leaves with adhering oogonia, while short vegetative tillers possessed neither inflorescences nor symptoms of downy mildew infection. Longitudinal and transverse sections of the tiller bases were cut either by hand with a razor blade or with the aid of a Reichert freezing microtome (sections 5 or 10 μm thick). Sections, however cut, were stained in lactophenol–trypan blue (1%) for 10 min then washed and mounted in lactophenol. The hyphae of *Peronosclerospora noblei* stained intensely blue, while the host tissue was only lightly stained. Special attention was paid to the spatial location of hyphae in axillary buds that were in various phases of their development on the tiller bases.

The methods used to determine the locations and morphology of the hyphae in various parts of the infected tillers depended on the characteristics of that tiller part. For the detection of hyphae in the growing points, nodes and internodes of infected tillers and in malformed inflorescences, tissues were fixed in 80% ethanol–acetic acid (1 : 1) for 24 h, dehydrated, wax-infiltrated, embedded and microtome-sectioned (sections 5 or 10 μm thick). Sections were stained with safranin and fast green (Clark 1973). A number of other staining schedules, e.g. chlorazol blue O, Flemming's triple stain, periodic acid-schiff, thionin and orange G and Weigert's haematoxylin (Johansen 1940; Clark 1973), were used, but safranin and fast green was found to give the best differentiation.

The location and morphology of hyphae in the leaf blades and sheaths was ascertained by either of two methods. The first method involved staining transverse free-hand or freezing microtome sections with lactophenol–trypan blue (1%) or safranin and fast green (Johansen 1940). The second method consisted of clearing and staining whole leaf pieces. A number of clearing methods, such as that of Shipton and Brown (1962) were tried initially, but all were found to be unsuitable for the leaves of *Sorghum leiocladum* that contain large amounts of silica. The method used during the study was adapted from O'Brien (1974). Pieces of leaf 1 cm long, either fresh or fixed in 80% ethanol–acetic acid (1 : 1), were cleared by placing them in 5 mL of a solution of 90% ethanol–80% lactic acid (1 : 1) in a 25-mL McCartney bottle. The capped McCartney bottles were autoclaved at 120°C, 103.4 kPa for 20 min. When cool, the leaf pieces were removed from the clearing solution and placed in 5 mL of a solution of 2 : 1 90% ethanol : lactophenol trypan blue (1%) in a 25-mL McCartney bottle. The bottles were autoclaved under the same conditions as those used for clearing. After the leaf pieces had cooled they were transferred to 100% (w/v) chloral hydrate for 24 h at room temperature (20–30°C), then mounted in lactophenol for microscopic examination. Even after the double autoclaving, the leaf pieces retained their structural integrity.

Observations and results

Tiller bases

Tussocks of wild sorghum are composed of tightly packed tiller bases at and just below ground level, from which tillers of several types arise. Repeated production of tillers from the tiller bases results in enlargement of tussocks. On the underground parts of tiller bases the nodes are so crowded that the internodes are scarcely distinguishable. In this zone, vascular strands of xylem, phloem and associated sclerenchyma branch and coalesce in an irregular manner. Parenchyma is restricted to small isolated pockets both near the periphery of the tiller bases and surrounding the vascular strands.

In tillers displaying symptoms of infection by *P. noblei*, hyphae can be found throughout the year in the tiller bases. The hyphae are thin-walled, coenocytic, branched and varying in thickness from 2 to 10 μm. They are intercellular in parenchyma tissue and lie approximately parallel to the vascular strands. Haustoria (simple, lobate or digitate and up to 6 μm wide) penetrate parenchyma cells and occasionally the tracheids of the vascular strands (Fig. 1*a*). Hyphae were never found in the tiller bases from which either flowering tillers or short vegetative tillers had earlier developed.

The developing tiller

At the beginning of a growing season, a number of tiller buds become active on the upper parts of tiller bases. Hyphae in the tiller bases grow into some of the tiller buds, but not into others. By the time hyphae invade the tiller buds, the primordia of the first two to four leaves have developed. Hyphae do not invade these developing leaves, which remain green and narrow. While these first-formed leaves are expanding and are 2–4 mm long, hyphae invade the apical meristem and the leaf primordia that subsequently develop. In the meristem, the hyphae are, for the most part, confined to the corpus, invading the tunica only at the points where leaf primordia are forming. Here the hyphae are thin-walled, coenocytic, rarely branching, 3–5 μm wide and intercellular in the parenchyma. Lobate haustoria, up to 3 μm wide, form in some of the parenchyma cells.

The first one or two leaf primordia entered by hyphae are, in most cases, not extensively permeated. Hyphae are concentrated in the basal portions of the leaf primordia, where there is an intercalary meristem. While new cells are being produced in the direction of the leaf tip, hyphal strands grow for a short distance towards the tip of the leaf primordia. When a leaf primordium reaches a length of 10–20 mm, a narrow band of small, compact parenchyma cells arises approximately halfway between the meristem at the base of the primordium and the apex of the primordium. A new intercalary meristem arises above this parenchyma band. The tissue between this meristem and the tip of the primordia becomes the leaf blade, while the tissue below it becomes the leaf sheath. Hyphae proliferate freely above the second meristem but remain sparse below it. The leaf blade lengthens by transverse division of cells in the intercalary meristem, while the leaf sheath lengthens by transverse divisions of cells in the lower meristem. Elongation of both the blades and sheaths is rapid in the early stages, and hyphal

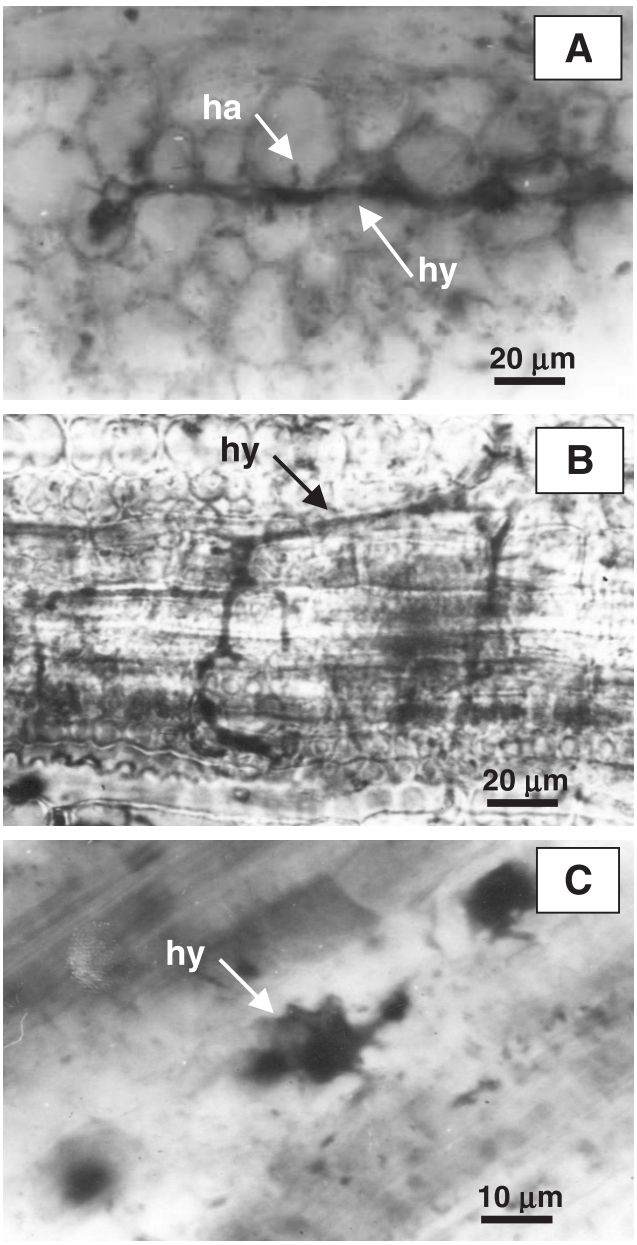

Fig. 1. Downy mildew on *Sorghum leiocladum*. (*A*) Hyphae and simple haustoria in base of infected tiller; (*B*) 'slender' hyphae in chlorotic leaf blade; (*C*) 'polymorphic' hyphae in chlorotic leaf blade. ha, haustorium; hy, hypha.

extension keeps pace with the elongation of the host cells. As the leaf blade expands, the hyphal strands grow throughout the mesophyll. The extent of invasion of the mesophyll depends on the position of hyphal strands across the width of the upper meristem. As a consequence, hyphae may be restricted to one side of a particular leaf blade, to isolated areas, or to the interveinal tissue. In the first one or two leaves invaded by the pathogen, hyphae are confined to the lower half of the blades.

The invaded areas of the leaf blades gradually become chlorotic before the blade is fully expanded. Chlorosis develops only in those areas that have been invaded by hyphae. Hyphae are never found in areas that remain green. In the leaf sheaths, individual hyphal strands may be present in the mesophyll, but the extent of spread is never as great as that which occurs in the blades.

In all the leaf primordia formed subsequent to the development of the first one or two invaded primordia, hyphae are much more profuse than in the earlier-formed primordia. The pattern of hyphal invasion is identical with that described above for the earlier-formed primordia, but hyphae move freely into the mesophyll tissue along the whole length of the blade. These leaves are entirely chlorotic before they emerge from the surrounding sheaths of the earlier-formed leaves. While the leaves are expanding, the hyphae are thin-walled, coenocytic, sparingly branched, 3–5 µm wide, intercellular in the mesophyll and tending to be parallel to the vascular bundles (Fig. 1*b*). Hyphae rarely invade the vascular bundle sheaths. With a single exception, only isolated hyphal strands were observed in leaf sheaths. In one of the leaf sheaths examined hyphae were found extensively in chlorotic areas immediately below the ligule.

Asexual sporulation occurs first at the distal end of chlorotic areas on invaded leaves, and the zone of sporulation increases in the direction of the base of the leaf blades in a stepwise fashion. In the part of the blade on which sporulation is occurring, two types of hyphae are present. The first type is identical with that found in expanding leaf blades and can be termed the 'slender' type (Fig. 1*b*). The second type, also found in the intercellular spaces, is extensively branched and 5–15 µm wide (Fig. 1*c*) and has been termed 'polymorphic'. Conidiophores develop from 'polymorphic' hyphae that have invaded the substomatal cavities. There are three types of haustoria, namely simple, lobate and digitate. They form in the cells of the mesophyll, epidermis and bundle sheath. Immediately behind the area of leaf blade, where asexual sporulation is occurring, the 'polymorphic' type is much more abundant than the 'slender' type. In the parts of leaves 1–2 cm in advance of where there is sporulation, both types of hyphae are present in approximately equal proportions. Towards the base of the blade, the proportion of 'slender' type hyphae increases, until near the leaf ligule only 'slender' type hyphae can be found.

Soon after an infected tiller reaches its maximum height, the last-formed, partly expanded, chlorotic leaves necrose rapidly from the tips towards the ligules. Ahead of the advancing necrotic area, the tissue becomes dark brown in a band 3–4 cm wide; this band will be termed the 'transition zone'. Just behind the advancing border of this zone, oogonia develop simultaneously across the whole width of the leaf blade. They are first evident as swellings in terminal or intercalary positions in the polymorphic hyphae. As the

oogonia enlarge within the intervascular areas, the parenchyma cells become more and more displaced. The vascular bundles and the upper and lower epidermis, which remain intact, tend to resist pressure from the expanding oogonia. As a result, the intervascular spaces become tightly packed with oogonia. The combined action of the wind and alternate wetting and drying of the necrotic leaves aids in the splitting of the upper and lower epidermis, finally resulting in the fraying of these leaves.

Nodes and internodes

The distribution of hyphae in the nodal region of culms is influenced considerably by the anatomy of the region. The anatomical features of the nodal region will be described before the location of the hyphae is discussed.

Immediately below the point of the leaf attachment at a node, there is a zone of lateral vascular bundles and vascular anastomosis, the nodal plexus, approximately 3 mm long. Immediately above the point of leaf attachment there is a zone of vascular anastomosis. From that zone, vascular bundles extend into the axillary bud. There is an intercalary meristematic zone about 1.5 mm above the point of leaf attachment in which there are flattened, polygonal, parenchyma cells, as well as juvenile vascular bundles lacking bundle sheaths and possessing only protoxylem vessels. Cells derived from this meristem comprise the tissue of the internodes.

In the nodes, hyphae are found predominantly in the intercalary meristem, where they are 3–7 μm wide and intercellular in the developing parenchyma. Hyphae are mostly close to the young vascular bundles and oriented approximately parallel to them. Simple and lobate haustoria, arising from the intercellular hyphae, form in the parenchyma cells. There is extensive spread of hyphae between the parenchyma cells of the nodal plexus, and to a lesser degree in the tissue adjacent to vascular bundles at the point of leaf attachment and in the lower parts of the leaf sheaths, where they join the node.

Evidence from longitudinal sections and serial transverse sections taken at various positions on the internodes of tillers indicated that internodal tissues are not heavily invaded by mycelium. Branching hyphae, 3–7 μm wide, occur only in discontinuous groups between cells of the parenchymatous ground tissue and occasionally in the vascular bundle sheath. Simple and lobate haustoria are present in some of the parenchyma cells. Hyphae follow a twisting path among the cells and in any section, longitudinal or transverse, only short lengths or even sections of hyphae are seen.

These patterns of hyphal distribution are characteristic of all the nodes and internodes of infected tillers, but considerably more hyphae are found in the nodes and internodes above the point of attachment of the uppermost normal basal leaf.

Tiller abnormalities

Two types of tiller abnormalities are observed on downy mildew-infected tillers. The first type consists of the bending and twisting of the upper parts of the culm and the associated leaves. Thin-walled, coenocytic hyphae, 2–5 μm wide are present in the leaves and culms of the abnormality and are in similar locations to the hyphae found in the leaves and culms of unproliferated infected tillers. The second type of vegetative abnormality consists of a number of crowded nodes at the apices of diseased tillers. From the nodes arise short, chlorotic leaves and 'root-like' structures. Histological investigations revealed that the anatomy of this region of closely arranged nodes resembles that of the lower parts of subterranean tiller bases. In this region, the hyphae are thin-walled, coenocytic and varying in width from 2 to 10 μm, and are intercellular in the parenchyma near the vascular strands. The slender and the polymorphic types of hyphae are found in the mesophyll of the short chlorotic leaves that arise from the nodes of the abnormality.

Abnormal inflorescences

Most infected tillers remained vegetative, but on a few tillers structures resembling inflorescences are found. These abnormal inflorescences are twisted, shorter than normal inflorescences and have a disorderly arrangement of branches and racemes. Spikelets are arranged in pairs, singly or in multiples up to five on the branches, or in some cases are attached directly to the main axis. The arrangement and composition of the spikelets are also markedly changed.

Hyphae are found in most parts of the malformed inflorescences. In the main axis, hyphae are most abundant in the nodal areas from which the spikelet structures arise, either directly or on pedicels. Hyphae also permeate the pedicels and the vegetative structures of the spikelet-like units. In all these tissues, hyphae are thin-walled, 4–10 μm wide and intercellular in parenchyma. In the reproductive organs, hyphae are found in the ovary wall, style and stigma and in the filaments and anther walls of stamens. The hyphae are 2.5–8.0 μm wide and rarely branched, except where they enter cells of the parenchyma tissue to form haustoria. Hyphal fragments were observed in the ovule of only one ovary of the 20 sectioned.

Roots

No hyphae were found in the roots arising from the tiller bases of either downy mildew-infected tillers or flowering tillers.

Discussion

Histological studies of the tiller bases from which individual flowering tillers and downy mildew-infected tillers, respectively, arise show that hyphae of *Peronosclerospora noblei* perennate in the tiller bases of infected tillers but not

in the bases of flowering tillers. The location and activity of these hyphae in the tiller bases during the early stages of tiller bud growth determines which tillers become infected. During the growing season of the grass, and more so during its dormant season, the whole or part of some tiller bases may die. Consequently, over a number of seasons an infected tussock may be separated into several smaller tussocks, the tillers of which may have hyphae in their bases. Some authors (Weston and Weber 1928; Thirumalachar and Narasimhan 1952; Semeniuk and Mankin 1964; Kenneth 1966) have suggested that downy mildews on several wild perennial grasses survive from one growing season to the next as hyphae in the underground tiller parts. However, none of these authors provided any evidence to support their suggestions.

On some tussocks of *Sorghum leiocladum*, downy mildew-infected tillers and flowering tillers occur together, a feature that has been recorded for other downy mildew–host combinations. Although Leece (1941) surmised that an uneven distribution of hyphae in sugarcane sets infected by *Peronosclerospora sacchari* (Miyake) Shirai & Hara was responsible for plants with healthy tillers and downy mildew-infected tillers, he did not provide any data to support his views. It has been shown in this study that the location and activity of hyphae in the tiller bases at the beginning of the growing season (when tiller buds are expanding) determines which tiller buds are invaded by hyphae of *Peronosclerospora noblei*. Invaded tiller buds grow on to become tillers that are systemically infected, while buds that escape invasion develop into flowering tillers or short vegetative tillers. Below ground, tussocks consist of a large number of separate tiller bases, so the relative numbers of infected tillers and flowering tillers produced each season may be a function of the location and activity of hyphae in all the tiller bases of a particular tussock.

Tillers of *Sorghum leiocladum* with symptoms of downy mildew bear two to four basal leaves of normal appearance. The lack of leaf symptoms until the third, fourth or fifth leaf has expanded is a consequence of delayed invasion of the apical meristem. That invasion does not occur until the first two to four leaf primordia have been found. A similar delay in symptom expression has been reported for other downy mildew–host combinations, e.g. *Peronosclerospora maydis* (Racib.) Shaw on maize (Inaba *et al.* 1980*a*), *P. philippinensis* (Weston) Shaw on maize (Weston 1923; Dalmacio and Exconde 1970) and *Sclerospora graminicola* (Sacc.) Schrot. on *Pennisetum americanum* (Suryanarayana 1952) and on *Setaria italica* (L.) Beauv. (McDonough 1938). In the first invaded leaf primordium, hyphae are restricted to the basal portion of the leaf blade that ultimately develops. This results in partial chlorosis of the leaf blade. In these leaf blades, there is apparently limited or no progression of the hyphae beyond the sites of initial establishment of the hyphae in the leaf primordium. Support for this concept comes from the observation that the chlorotic areas do not increase in size during leaf expansion. In addition, hyphae are never found in the green areas of partly chlorotic leaf blades. Dalmacio and Exconde (1970) also found that hyphae of *P. philippinensis* were restricted to the chlorotic areas of leaf blades of maize.

The presence of hyphae in the abnormal structures that are sometimes found on tillers, has implications with respect to the perennation of the fungus. Hyphae were found in the basal parts of one form of tiller abnormality, namely the one that consisted of a number of closely arranged nodes from which short chlorotic leaves and root-like structures arose. This entire abnormality could develop into a mature tussock if it were to be carried to the ground and take root there. Within one spikelet-like structure of an abnormal inflorescence, hyphae were found in the ovule of the ovary. If this caryopsis had matured and later germinated, a systemically infected seedling may have developed. However, only a very small proportion of infected tillers produced inflorescence-like structures, and mature caryopses were never found in any of the abnormal inflorescences. Taking these facts into account, it is highly unlikely that hyphae in caryopses of *Sorghum leiocladum* play a role in the perennation of *Peronosclerospora noblei*.

Two types of hyphae were found in the invaded organs of downy mildew-infected tillers. 'Slender' hyphae were found in tiller bases, meristematic zones, nodes, internodes, abnormal tiller and inflorescence structures, and in those portions of leaf blades where asexual sporulation had not occurred. Hyphae of the second type, 'polymorphic', were found only in the parts of the leaf blade supporting, or that had previously supported, asexual sporulation and later sexual sporulation. The two types of hyphae may have different biological purposes, the slender type in vegetative growth and the polymorphic type in the reproduction of the downy mildew. These results confirm the observations of other authors, e.g. Payak *et al.* (1970) and Inaba *et al.* (1980*b*), who considered that hyphae in the leaf blade change from the slender type to the polymorphic type as a precursor to asexual and sexual sporulation.

The data presented in this paper show that the location and activity of hyphae of *Peronosclerospora noblei* in tiller bases and developing tillers of wild sorghum are responsible for the perennation of the fungus and the expression of symptoms in infected tillers. For other downy mildew–perennial grass combinations, hyphae of the pathogen may play similar roles as those described above for *Peronosclerospora noblei* on *Sorghum leiocladum*.

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