Goldacre Review

The mixotrophic nature of photosynthetic plants

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Abstract. Plants typically have photosynthetically competent green shoots. To complement resources derived from the atmospheric environment, plants also acquire essential elements from soil. Inorganic ions and molecules are generally considered to be the sources of soil-derived nutrients, and plants tested in this respect can grow with only inorganic nutrients and so can live as autotrophs. However, mycorrhizal symbionts are known to access nutrients from organic matter. Furthermore, specialist lineages of terrestrial photosynthetically competent plants are mixotrophic, including species that obtain organic nutrition from animal prey (carnivores), fungal partners (mycoheterotrophs) or plant hosts (hemi-parasites). Although mixotrophy is deemed the exception in terrestrial plants, it is a common mode of nutrition in aquatic algae. There is mounting evidence that non-specialist plants acquire organic compounds as sources of nutrients, taking up and metabolising a range of organic monomers, oligomers, polymers and even microbes as sources of nitrogen and phosphorus. Plasma-membrane located transporter proteins facilitate the uptake of low-molecular mass organic compounds, endo- and phagocytosis may enable the acquisition of larger compounds, although this has not been confirmed. Identifying the mechanisms involved in the acquisition of organic nutrients will provide understanding of the ecological significance of mixotrophy. Here, we discuss mixotrophy in the context of nitrogen and phosphorus nutrition drawing parallels between algae and plants.

Additional keywords: endocytosis, mixotrophy, organic nutrients, plant nutrition, root hairs.

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Introduction

Terrestrial primary production and the resulting vast phytomass is fuelled by the photosynthetic power of the plant shoot, complemented by the root's acquisition of water and nutrients from soil. Although most terrestrial organisms live predominantly either above- or belowground, plants, with the notable exception of epiphytes, span both. Green plants are considered autotrophs that are capable of converting inorganic material into organic matter using the electromagnetic energy of light. The atmosphere provides the substrates for photosynthesis, whereas all other essential elements are predominantly derived from soil (Marschner 1995). A plant is truly autotrophic when its roots acquire only inorganic nutrients, including nitrate, ammonium, base cations, ortho- and polyphosphates and sulfate. Autotrophy has been shown in hydroponic experiments on the essentiality of particular elements in which plants complete their life cycle in the absence of exogenous organic compounds (Arnon and Stout 1939; Marschner 1995; Epstein and Bloom 2005). However, roots in soil are exposed to numerous organic compounds, and much of the soil's nitrogen (N) and phosphorus (P) is organic.

Soil N occurs predominantly as amino acid-based molecules (Schulten and Schnitzer 1997), organic P, for example, as phytate and nucleotides, and organic sulfur as a variety of organosulfur compounds (Dyer and Wrenshall 1941; Schulte and Kelling 1999). In addition, soils contain a large range of carbon compounds such as organic acids and sugars, including those released by roots, and there are bidirectional fluxes (soil–roots) of organic matter (Jones *et al.* 2009).

There is broad agreement that plants acquire N and P in inorganic form. Inorganic N is relatively mobile in soil, although orthophosphate has significant restrictions on its mobility, but has greater mobility than phytate or polyphosphate. The acquisition of inorganic ions and molecules by roots involves plasma membrane located transporters that catalyse selective uptake, and accumulation, of inorganic N and P. It is generally assumed that plants are net exporters of organic compounds into soil and importers of inorganic ions and molecules, but recently the role of organic compounds as plant nutrients is being re-evaluated (reviewed by Paungfoo-Lonhienne *et al.* 2012).

It therefore seems timely to explore mixotrophy – the combined nutritional modes of autotrophy and heterotrophy – in ecological and mechanistic contexts. We focus here on N and P because they occur as organic and inorganic compounds in soils, and their supply is a major determinant of plant growth in natural ecosystems and bioproduction systems (reviewed by Lambers *et al.* 2008; Peltzer *et al.* 2010; Ma *et al.* 2012). We briefly outline nutrient acquisition strategies to highlight root specialisations, and discuss mixotrophy in algae and in plants. The last section discusses how larger organic molecules may enter roots, and the function of organic compounds as signalling molecules that increase root branching and the presence of root tips and associated root hair zones.

Overview of nutrient acquisition strategies

Root specialisations: mycorrhizal fungi, N_2 fixing procaryotes and cluster roots

The four main specialisations for nutrient acquisition are root exudates and their extreme occurrence in the functioning of cluster roots, and symbioses with mycorrhizal fungi and N_2 fixing bacteria (Fig. 1). Although exudates universally contribute to root function, their amount and composition varies among species and growth situations, the other specialisations are species specific. The mycorrhizal symbioses are most common and formed by most of plant species with a range of fungal partners, different extent of mycorrhizal colonisation, and varying benefits derived from the symbiosis. The dependence of plants on mycorrhizal fungi for accessing nutrients from organic matter and soil minerals is well established (Smith and Read 2008). Mycorrhizas are not the focus here,

although we discuss mycoheterotrophy in the context of green orchids.

An estimated 10% of plant taxa have N_2 fixing symbioses (Franche *et al.* 2009), providing an alternative N source to soil N at the cost of photosynthates required to operate N_2 fixation by the bacterial symbionts and, especially, the production and maintenance of the specialised structures (nodules, rhizothamnia and coralloid toots) housing the symbiont. Far fewer plant species have N_2 fixing symbioses than mycorrhizas, although most ecosystems contain N_2 fixing plants. N_2 fixing symbioses are beyond the scope of this review (see Sprent 2001).

Several thousand plant species do not form mycorrhizal symbioses, including all species of the Proteaceae, many sedges, and species in several other plant families (Tester *et al.* 1987; Lambers *et al.* 2008), including the model plant *Arabidopsis thaliana* and other Brassicaceae. However, the recently discovered symbiotic associations with endophytic dark septate fungi are quite common (Caldwell *et al.* 2000), and occur in *Arabidopsis thaliana* (Mandyam *et al.* 2013). Most Proteaceae, many sedges, some N_2 fixing species (perhaps most actinorhizal species and some legumes) and other species produce cluster roots in response to low nutrient availability. Cluster roots are considered particularly beneficial in P-depauperate soils (reviewed by Dinkelaker *et al.* 1995; Neumann and Martinoia 2002; Lambers *et al.* 2006). Cluster roots impact a small volume of soil by exuding protons, organic acids, secondary compounds and enzymes (Dinkelaker *et al.* 1995; Shane and Lambers 2005; Paungfoo-Lonhienne *et al.* 2009), and, less well characterised, through interaction with microbes (Marschner *et al.* 2002; Weisskopf *et al.* 2011).
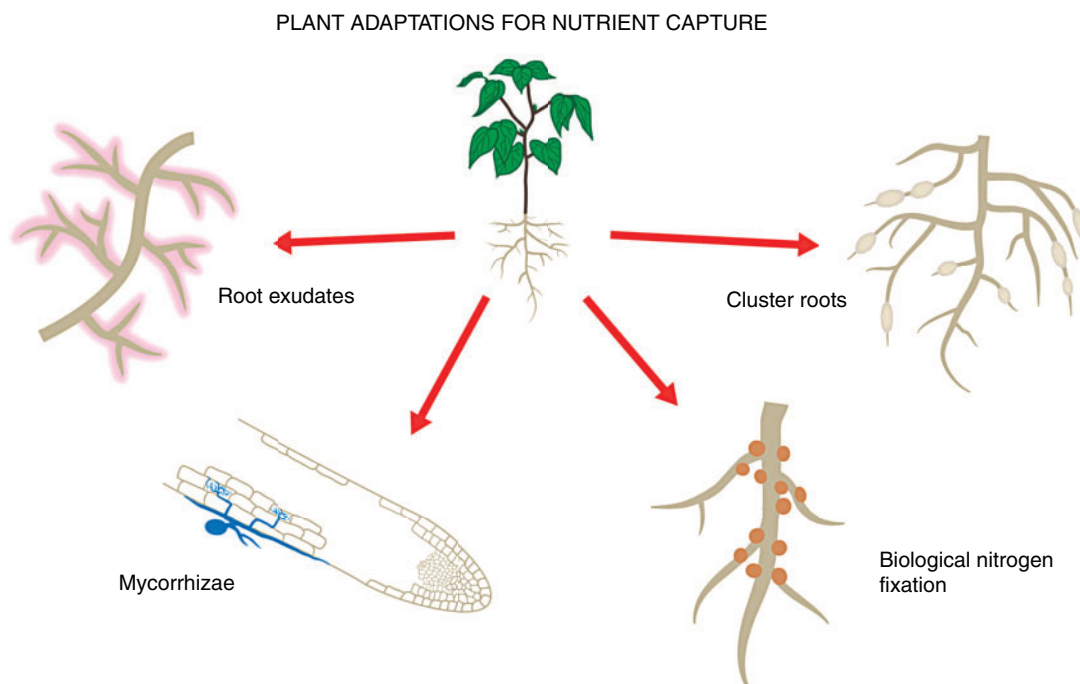
PLANT ADAPTATIONS FOR NUTRIENT CAPTURE

Fig. 1. Overview of plant strategies for nutrient acquisition. Four acknowledged strategies are (i) root exudates, (ii) mycorrhizal associations, (iii) N_2 fixing symbioses in leguminous and actinorhizal species, (iv) cluster roots.

Ecosystems have been classified along a gradient of 'organic-to-inorganic nutrients dominated', with the former requiring greater specialisation by the plant to access nutrients than the latter. In slow-mineralisation organic-nutrient rich ecosystems, such as heathlands and boreal forests, specialised ecto- and ericoid mycorrhizal fungi depolymerise organic matter via extracellular enzymes, and supply nutrients to plants (Read and Perez-Moreno 2003; Lambers *et al*. 2008). In rapid-mineralisation ecosystems such as grasslands, plants are have mostly arbuscular mycorrhizas and are thought to acquire inorganic nutrients that are derived from breakdown of organic matter by soil microbes (Read and Perez-Moreno 2003).

In light of all root specialisations, it remains uncertain what proportion of N and P is acquired by roots as inorganic or organic compounds. The difficulties associated with studying rhizosphere processes, including chemical characterisation of nutrients and quantification of their fluxes, have limited progress in understanding the importance of organic N and P for plant nutrition. However, roots take up a variety of organic compounds (Näsholm *et al*. 2009; Paungfoo-Lonhienne *et al*. 2012). An important consideration for the discussion on mixotrophy is that irrespective of root specialisation, a significant proportion of the root surface is free of fungi or other microbes (Bais *et al*. 2006).

Root exudates

Root exudates affect the availability of nutrients directly or indirectly (reviewed by Walker *et al*. 2003; Bais *et al*. 2006; Jones *et al*. 2009). Exudates are complex chemical mixtures of low and high molecular weight compounds, including organic acids, phytosiderophores (only in the Poaceae), sugars, vitamins, amino acids, purines, nucleosides, inorganic ions, dissolved gases, high molecular weight carbohydrates (mucilage), enzymes and root border cells (reviewed by Dakora and Phillips 2002). Exudates are communication agents and facilitate nutrient acquisition through various mechanisms (see reviews by Walker *et al*. 2003; Bais *et al*. 2006). The mechanisms that plants use to interpret the chemical signals in the rhizosphere that originate from soil organisms and other plants, remain largely unknown (Bais *et al*. 2006). This has relevance to mixotrophy, and is discussed below.

Root-derived enzymes released into the apoplast and rhizosphere may contribute to mixotrophy if they generate organic compounds that are subsequently acquired by roots. Root exoenzymes have been studied for their role in catalysing the breakdown of large organic molecules as well as other functions. High-molecular mass inorganic compounds (e.g. poly-phosphates) and organic compounds (e.g. protein, phytate, DNA) can be degraded by root-derived phosphatases, proteases, phytases and DNAses (reviewed by Paungfoo-Lonhienne *et al*. 2012). Phosphatases are exuded, for example, from cluster roots of Proteaceae and Fabaceae (Dinkelaker *et al*. 1995; Yamamura *et al*. 2002; Wasaki *et al*. 2003). In legumes, root phosphatase activity is higher than in other forbs regardless of the presence of nodules (Venterink 2011), and soil associated with N_2 fixing plants has higher extracellular phosphatase activity than soil without N_2 fixers (Houlton *et al*. 2008). Root-derived proteases have been described in *Hakea*

(Proteaceae), carnivorous plants, *Arabidopsis* and other species including crops (Adlassnig *et al*. 2012). Proteases are present in the root apoplast (Tornero *et al*. 1996; Hamilton *et al*. 2003), and the presence of protein degradation products in the apoplast of inner root cortex suggests that proteases are active in the apoplast and at the root surface (Paungfoo-Lonhienne *et al*. 2008).

From an evolutionary viewpoint, the release of enzymes into the rhizosphere is advantageous only if the nutrient gain outweighs the nutrient loss in the exudates, or alternatively, if breakdown products have important signalling roles (Paungfoo-Lonhienne *et al*. 2008). The presence of enzymes in the apoplast may catalyse the degradation of larger molecules that enter roots via transpiration-driven movement of the soil solution and diffusion, but this is not well characterised. Overall, there has been comparatively little research on root exoenzymes for plant nutrition, as it was generally assumed that microbes drive the breakdown of soil organic compounds. The rhizosphere was treated as a 'black box' and researchers were satisfied to consider microbial activity in general terms (Silberbush 2013). Recent advances in molecular methods characterising microbial communities in soil, rhizosphere and roots promise to advance knowledge of rhizosphere dynamics (Bulgarelli *et al*. 2012; Lundberg *et al*. 2012).

The positive relationship between the release of enzymes and access to organic nutrients that has been observed in plants tested in controlled conditions has not always been confirmed in soil. For example, wheat genotypes with greater exudation of phosphatase acquired more P from organic P when grown axenically, but not when grown in soil (George *et al*. 2008). Similarly, transgenically-enhanced root exudation of phytase improved plant growth with phytate in controlled conditions, but to a lesser extent in soil (reviewed by Richardson *et al*. 2009). Root-derived exoenzymes may have numerous roles, although how much they contribute to the breakdown of organic compounds in the apoplast relative to the rhizosphere, and subsequent uptake of the resulting compounds, is unknown.

Mixotrophy in phytoplankton, other aquatic organisms and terrestrial plants

Mixotrophy, as phagomixotrophy, is a common and biogeochemically important strategy in eukaryotic phytoplankton and a range of planktonic and benthic protists and metazoans which have acquired photosynthesis by endosymbiosis of cyanobacterial or microalgal cells or through kleptoplasty (Raven 1997; Stoecker 1998; Raven *et al*. 2009; Esteban *et al*. 2010; Hartmann *et al*. 2012; Flynn *et al*. 2013). In most planktonic habitats the strategy predicted to maximise the abundance of mixotrophs is a predominantly photosynthetic mode of energy gain in combination with consuming bacteria and other microbes which increases acquisition of essential elements (Crane and Grover 2010), thereby outcompeting planktonic phototrophs and phagotrophs (Tittel *et al*. 2003; Hartmann *et al*. 2012).

Is mixotrophy an ancestral state in algae? Endocytosis, itself a synapomorphy of eukaryotes, was how all extant algae and hence embryophytic ('higher') plants, regardless of whether or not they exhibit mixotrophy, obtained their plastids by primary,

secondary or tertiary endosymbiosis followed by genetic integration (Raven 1997; Raven *et al.* 2009). It is likely that phagomixotrophy was retained in some clades after the genetic integration of plastids (Raven *et al.* 2009). Phagotrophy also underlies kleptoplasty, where plastids from an algal food item are retained in a functional form in an otherwise heterotrophic cell (Raven *et al.* 2009; Flynn *et al.* 2013). Such kleptoplastids are capable of long-term functionality if the nucleus from the plastid source is retaining a functional state with the plastids which occurs in the ciliate *Myrionecta rubra* (Johnson *et al.* 2007). However, in some cases, plastid longevity occurs without retention of food alga nuclei or prior horizontal gene transfer from the algal nucleus to the grazer nucleus (Wagele *et al.* 2011; Pillet and Pawlowski 2013).

In addition to phagomixotrophy, most algae are also capable of osmomixotrophy, i.e. uptake of individual soluble organic molecules by transporters in the plasmalemma (Raven *et al.* 2009; Flynn *et al.* 2013) and perhaps, of soluble macromolecules by fluid-phase endocyotosis, so-called pinocytosis. The significance of osmomixotrophy in phytoplankton under natural conditions is unclear; it is possible that the net flux of low molecular mass organic solutes is outwards under many conditions (see Flynn *et al.* 2013). Osmomixotrophy also occurs in photosynthetic bacteria such as cyanobacteria, as well as in proteobacterial anoxygenic photosynthetic bacteria. Particularly widespread in the surface ocean are anoxygenic aerobic photosynthetic proteobacteria, and the otherwise heterotrophic proteobacteria with proteorhodopsin (Zubkov 2009; Raven and Donnelly 2013). Here the photochemical reactions do not power autotrophic CO_2 assimilation, but spare the use of respiratory processes in energising solute transport and ADP phosphorylation, and so may legitimately be considered mixotrophs since the cells use two sources of energy, i.e. electromagnetic radiation and dissolved organic compounds (Zubkov 2009; Raven and Donnelly 2013).

Osmomixotrophy as a source of organic carbon permitting growth (in the dark) or increasing growth (in the light) is not universal in autotrophs. Obligate photoautotrophy, i.e. the inability to grow other than with light as the energy source and inorganic nutrients supplying essential elements, has been reported for several algae (including cyanobacteria) (Raven 2012). As with algae that cannot be grown in laboratory culture under any conditions so far used, so with obligate photoautotrophic algae: perhaps the next culture attempt with different conditions will allow growth (for so far unculturable organisms) or growth with a contribution from external organic carbon (for obligate photoautotrophs). However, for the moment at least there are obligately photolithotrophic algae (Raven 2012). It is important to note that the obligate photoautotrophy does not preclude the assimilation of exogenous organic compounds, just their involvement in growth (Raven 2012). A final point about obligate photoautotrophy is that technical complications mean that it is not readily tested for embryophytic, and especially vascular plants.

An endocytosis-like uptake of protein by the planktonic heterotrophic planctomycete bacterium *Gemmata obscuriglobus* was recently reported (Lonhienne *et al.* 2010). This process is probably not involved in mixotrophy in anoxygenic photosynthetic bacteria, since the planctomycetes have a uniquely complex cell structure for a bacterium, and no planctomycete is known to have photosynthesis (Fuerst *et al.* 1993). However, a planctomycete contains proteorhodopsin (McCarren and DeLong 2007), so the endocytosis-like means of protein uptake could be involved in this variant on mixotrophy. In summary, the categorisation of autotrophic phytoplankton and heterotrophic microzooplankton does not account for the dual mode of nutrition and warrants revision of the dichotomous concept (Flynn *et al.* 2013).

A related process, which overlaps with mixotrophy phylogenetically and functionally, is auxotrophy – the inability of some algae to make all enzyme cofactors known as vitamin in metazoan nutrition. The most widespread vitamin requirement is that of cobalamin (vitamin B_{12}), with 155 of the 306 algae examined needing an external supply of vitamin B_{12} (Croft *et al.* 2006). Where the vitamin B_{12} auxotrophs are also phagomixotrophs the prey is presumably a source of the vitamin, bearing in mind that only archaea and bacteria are able to synthesise vitamin B_{12}. Auxotrophic algae that are not phagomixotrophs must obtain the vitamin from associated bacteria or archaea or from the growth medium. Vitamin B_{12} dependence comes about from loss of an alternative, vitamin B_{12}-independent, pathway of methionine synthesis (Helliwell *et al.* 2011). For other vitamins, auxotrophy comes about from loss of the capacity to synthesise the vitamin. Since embryophytic plants have no known requirement for exogenous vitamins, the assumption is that they evolved from a charophycean green alga with no requirements for exogenous vitamins. This is the most economical hypothesis, but it does not rule out the alternative of an auxotrophic algal ancestor with subsequent horizontal gene transfer that overcame the need for external vitamins.

Mixotrophy has been a recent focus of phytoplankton research (e.g. Bronk *et al.* 2007; Gómez-Baena *et al.* 2008; Zubkov *et al.* 2008; Poretsky *et al.* 2010; Flynn *et al.* 2013), and photosynthetic plants are also investigated for their ability to satisfy energy and nutritional needs with organic carbon (C) compounds. An obvious parallel to algal phagomixotrophy in green plants is the carnivorous mode of nutrition (Fig. 2; Raven *et al.* 2009). We do not consider carnivorous angiosperms in detail here, but note that they are photosynthetic (Juniper *et al.* 1989; Raven *et al.* 2009; Król *et al.* 2012), can take up organic C in amino acids and peptides via transporters (Schulze *et al.* 1999) and via endocytosis (Adlassnig *et al.* 2012), but have a significant C cost in trap construction and maintenance and in enzyme secretion (Ellison and Adamec 2011; Sirová *et al.* 2011).

Another variant on mixotrophy is the mycoheterotrophy in photosynthetically competent plants. It was originally thought that mycoheterotrophy only occurs in specialist achlorophyllous plants that obtain all their C and N from mycorrhizal fungi (Leake 2004; Leake and Cameron 2010). More recently it was discovered that some green orchids are mixotrophs, gaining organic C and N via a combination of mycoheterotrophy and photosynthesis (Fig. 2; reviewed by Selosse and Roy 2009). Substantial fungus-mediated mixotrophy occurs in the photosynthesising ericaceous tribe Pyroleae (Tedersoo *et al.* 2007) and the orchid *Cephalanthera damasonium* (Julou *et al.* 2005) with an estimated 10–67% of carbon obtained from fungal partners. Selosse and Roy (2009) draw analogies between mixotrophy of plants and algae, and suggest that partial mycoheterotrophy may be more
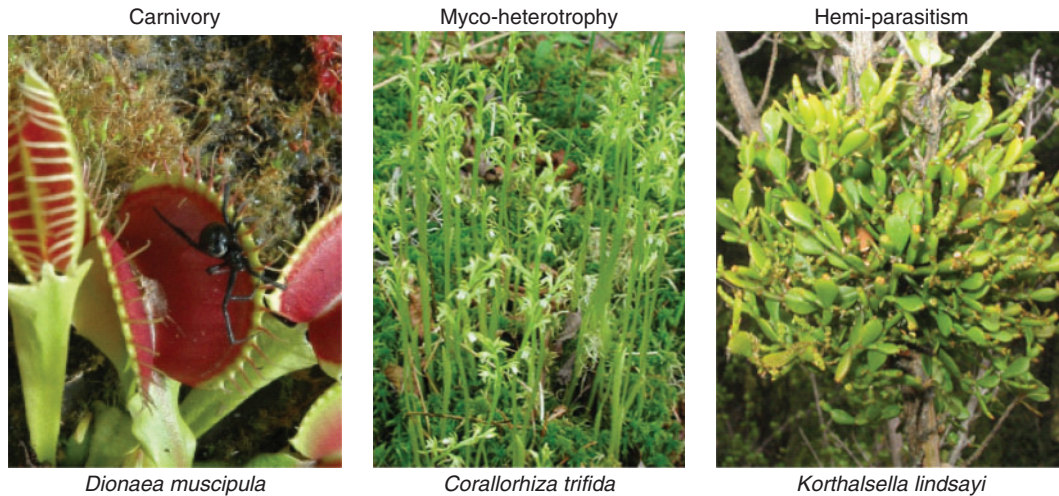
Carnivory

Dionaea muscipula

Myco-heterotrophy

Corallorhiza trifida

Hemi-parasitism

Korthalsella lindsayi

Fig. 2. Acknowledged mixotrophic strategies of green plants. Animal prey, fungi or host plants provide organic nutrients to carnivorous, partially myco-heterotrophic and hemiparasitic photosynthesing plants (photograph courtesy of Jeremiah Harris, Leilani Nepenthes, Colorado Springs CO (*Dionaea muscipula*), Eleanor Saulys and the Connecticut Botanical Society (*Corallorhiza trifida*), and John Barkla, NZ Plant Conservation Network (*Korthalsella lindsayi*) and reproduced with permission.

common in light-limited environments than currently acknowledged. The authors also highlight the dearth of knowledge of cellular mechanisms enabling the transfer of nutrients between fungi and partially mycoheterotrophic plants.

Analogous to mycoheterotrophy by photosynthetically competent plants, but without a fungal link, is hemiparasitism (Fig. 2; Marshall and Ehleringer 1990). Here a photosynthetically competent vascular (usually angiosperm) plant obtains all water and nutrient elements which cannot be supplied by its own photosynthesis from another (usually angiosperm) vascular plant (Raven 1983). Hemiparasites are generally linked exclusively to the host xylem and acquire N mostly as organic N as all root-acquired, including organic N, ammonium and N_2 fixed by root nodules or rhizothamnia, move in the xylem as organic N, although only nitrate and most P move as inorganic constituents (Raven 1983). Acquisition of organic C and N by hemiparasites from the xylem stream of the host involves the parallel acquisition of two or more atoms of organic C per atom N when dicarboxylic amino acid and their amides are the form in which N is moved, and one C per N (with not all C recoverable as organic C), when ureides such as allantoin are the main N transport molecules.

Mixotrophy is deemed the exception in terrestrial plants, excepting carnivorous, partial mycoheterotrophic or hemi-parasitic plants, but a recent study using ^{13}C-labelled litter showed that *Quercus petraea* trees in north-eastern France are mixotrophic in spring (Bréda *et al*. 2013).

Organic nutrients as sources of essential nutrients for plants

In recent decades it has been 'rediscovered' that nitrate and ammonium are not the sole N sources for plants; early plant nutrition researchers considered a surprising variety of organic compounds as nutrient sources (reviewed by Paungfoo-Lonhienne *et al*. 2012). Amino acids are present in soil, and plants use or even prefer amino acids over other N forms (Chapin *et al*. 1993; Chapin 1995; Näsholm *et al*. 1998; Schimel and Bennett 2004), and amino acid transporters catalysing the uptake of amino acids into roots have been identified (Hirner *et al*. 2006; Lee *et al*. 2007; Svennerstam *et al*. 2007, 2011). The difficulties associated with quantifying N conversions in soils, detecting simultaneous uptake and release of N by soil and symbiotic organisms and roots, and identifying the chemical form in which N is acquired by plants have prevented unequivocal assessment of the contribution of organic N to the plants' N uptake (reviewed by Näsholm *et al*. 2009). Ecological and physiological studies have evaluated amino acids for their role as organic N and C sources, but debate continues as to whether these compounds are generated at sufficient rates in soil to be significant N sources, and whether plants can compete with microbes for their uptake. Larger organic N compounds in soils include peptides and proteins. Membrane transporters catalysing the uptake of di- and tri-peptides into roots have now been identified (Komarova *et al*. 2008). It has been suggested that use of peptides as N and C source contributes to the success of the endemic angiosperm *Deschampsia antarctica* with increasing temperatures on the Antarctic Peninsula (Hill *et al*. 2011). We found that green fluorescent protein enters root hairs and supplements inorganic N in low-N supplied *Arabidopsis* but have not identified the mechanisms that permit such uptake (Paungfoo-Lonhienne *et al*. 2008).

Experimental and technical innovations are advancing knowledge on organic nutrients and include microdialysis as tool to quantify N flux via passive diffusion (Inselsbacher and Näsholm 2012). Soil microdialysis showed that amino acids account for 80% of the soil N supply in undisturbed boreal forest soil, whereas conventional extraction techniques detected inorganic N as the dominant soil N form. Microdialysis and analysis of a larger range of soil organic

compounds have great potential to improve knowledge of N and other nutrients (Inselsbacher *et al.* 2011; Warren 2013), as widely used soil extraction methods were established to identify fertiliser needs in agriculture focussed on inorganic nutrients. Promising techniques include high resolution research techniques of soil, roots and soil microorganisms including confocal and nano-SIMS imaging with fluorescent and stable isotope labelled N compounds (Paungfoo-Lonhienne *et al.* 2008; Clode *et al.* 2009; Whiteside *et al.* 2009; Kilburn *et al.* 2010).

Similar questions as for N arise about the role of organic P for plant nutrition. Inorganic P (orthophosphate, P_i) is considered the main source for plants and is acquired via membrane transporters (Mudge *et al.* 2002; Smith *et al.* 2003). Organic P forms are generally considered unavailable for plant uptake, but have been more recently investigated as sources for plants. As discussed above, exoenzymes cleaving phosphate esters have been described in several plants species, and the ability of axenically cultivated *Arabidopsis* and wheat to grow with nucleic acid and other organic P forms as sole P sources was thought to be due to exuded DNAases, phytases and phosphatases (Chen *et al.* 2000; Richardson *et al.* 2000). We showed that organic P consisting of 25-nucleotide DNA, that was protected from enzymatic degradation, entered root cells (Fig. 3; Paungfoo-Lonhienne *et al.* 2010*a*), indicating that organic P compounds can enter roots. So far there have been no reports that angiosperms can use the uptake system of some cyanobacteria and certain other bacteria which acquire and then cleave organic phosphonates (Dyhrman *et al.* 2006).

Possible mechanisms of the uptake of large organic compounds into roots

Adding to the uncertainties of the role of organic nutrients is the scant mechanistic understanding of how large organic compounds enter roots. The membrane-spanning proteins that catalyse selective passage of ions or molecules across the plasma membrane cannot transport large macromolecules such as proteins, polynucleotides or polysaccharides. Eukaryotic cells ingest macromolecules via endocytosis by progressively enclosing the substance in a section of the plasma membrane, followed by invagination and pinching off to form an intracellular vesicle containing the ingested substance (Alberts *et al.* 1989). Endocytosis retrieves membrane material and associated cargo from the plasma membrane for internal utilisation or destruction, and for processing and recycling to the plasma membrane (Robinson 2005). Endocytosis is common to all eukaryotes and enabled by cytoskeleton-forming proteins, but was thought not to occur in prokaryotes due to missing cellular components and thick and rigid cell walls. However, uptake of proteins by bacterium *Gemmata obscuriglobus* (Planctomycetes) occurs as an energy-dependent process analogous to eukaryotic endocytosis (Lonhienne *et al.* 2010), and some bacteria incorporate other bacteria as endosymbionts (McCutcheon and von Dohlen 2011).

Endocytosis serves multiple purposes in plants including membrane recycling, protein transport, cell-to-cell communication, cell signalling and cell wall morphogenesis (Holstein 2002; Šamaj *et al.* 2004; Šamaj *et al.* 2005; Geldner and Jurgens 2006). The role of endocytosis for nutrient uptake
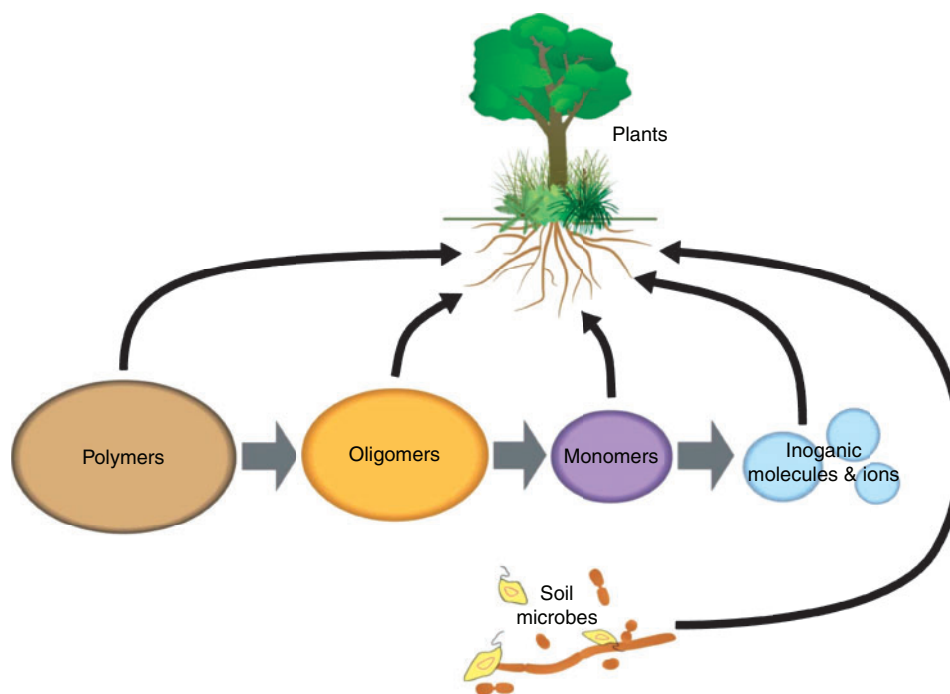

Fig. 3.   Proposed scheme of plant nutrient sources in which soil microbes and root-derived enzymes contribute to depolymerisation and mineralisation of organic matter, and plants acquire not only inorganic nutrients directly and organic nutrients indirectly via mycorrhizal symbionts, but also take up a suite of organic compounds and microbes (modified from Paungfoo-Lonhienne *et al.* 2012).

remains a controversial topic. In the 1960s pinocytosis, a type of endocytosis that involves invagination of the plasma membrane around a fluid phase and subsequent formation of a small versicle within the cytoplasm, was considered by some researchers as a main route of entry of nutrients into root cells (Jensen and McLaren 1960; Bradfute *et al.* 1964; Bradfute and McLaren 1964; reviewed by Cram 1980). Cram (1980) critically reviewed this hypothesis, pointing out major problems such as the required implausibly high density of selective binding sites in the membrane and the thermodynamic and mechanistic difficulties of creating a fluid-filled spaces by invagination of the plasmalemma in a turgid plant cell. The points raised by Cram (1980) were reiterated and developed by Raven (1987) and Robinson *et al.* (2008). However, FM dyes, the most reliable endocytosis tracer (Emans *et al.* 2002; Bolte *et al.* 2004), have unequivocally demonstrated that plant cells perform endocytosis (Emans *et al.* 2002) and contain endosomes (Geldner 2004; Voigt *et al.* 2005). Membrane internalisation in plant cells occurs principally via clathrin-coated vesicles similar to most animal cells (Pérez-Gómez and Moore 2007). Even though the analysis of the *Arabidopsis* genome has revealed considerable conservation of components of the endocytotic machinery among eukaryotes, endocytosis research in plants has lagged behind that of animals. A proposed reason has been the lack of obvious candidates for endocytotic cargo molecules (Robinson *et al.* 2008).

There is evidence for a vesicle-mediated transport system between vacuole and plasma membrane (Echeverria 2000). Internalised lucifer yellow, an endocytosis marker, occurs in transition zone cells of the inner cortex of intact maize root apices indicating that large molecules are transported across cortex cells (Baluska *et al.* 2004, who acknowledged that results from the use of lucifer yellow can be compromised by the occurrence of carrier-mediated movement of this tracer across plant membranes). That larger molecules enter roots via endocytosis has long been proposed as exogenously supplied macromolecules such as DNA were detected in roots (reviewed by Paungfoo-Lonhienne *et al.* 2012). Sucrose and glucose enter cells by membrane transporters (Sauer 2007) and are also incorporated into intact walled plant cells via endocyctosis (Etxeberria *et al.* 2005*a*, 2005*b*). Endocytosis and an additional unknown mechanism have been proposed to facilitate entry of peptides into walled plant cells (Eggenberger *et al.* 2011).

It was assumed that carnivorous plants first digest organic compounds in animal-catching organs such as pitchers into inorganic and smaller organic compounds (amino acids, peptides), before transport into plant cells by membrane transporters (Schulze *et al.* 1999). Recently, endocytosis was identified as a mechanism facilitating entry of proteins into tissues of carnivorous plants. Proteins are absorbed by endosomes which fuse with primary lysosomes which results in larger compartments where degradation takes place (Adlassnig *et al.* 2012). Endocytosis also plays an integral role in the endocytic uptake of *Rhizobium* bacteria in legume roots, involving internalisation into plant cells (reviewed by Brewin 2004).

Microbes occur in much higher density in the rhizosphere than bulk soil and are attracted by root-derived compounds and

debris, and it is conceivable that plants acquire and digest microbes as sources of nutrients. We tested this possibility and presented the first evidence that root cells incorporate non-pathogenic and non-symbiotic microbes as nutrient sources (Fig. 3; Paungfoo-Lonhienne *et al.* 2010*b*). As microbes compete with plants for soil nutrients, uptake and digestion of microbes could be an adaptation for securing nutrients (Paungfoo-Lonhienne *et al.* 2010*b*).

Effect of organic nutrients on root morphology

It is possible that the effects of external organic compounds on root function are related to mixotrophy. For example, organic compounds that trigger increased root branching by increasing the number of root tips and extent of root hair production, may stimulate processes that rely on the presence of these structures especially nutrient uptake. Indeed, the observed effects of organic polymers on root morphogenesis include enhanced root branching and root hair growth (Fig. 4) and are congruent with the notion that organic polymers facilitate root proliferation in sites that are rich in organic matter (Paungfoo-Lonhienne *et al.* 2008; Paungfoo-Lonhienne *et al.* 2010*a*).

An extension of the root system allows exploration of soil for the continued absorption of inorganic and organic nutrients. The
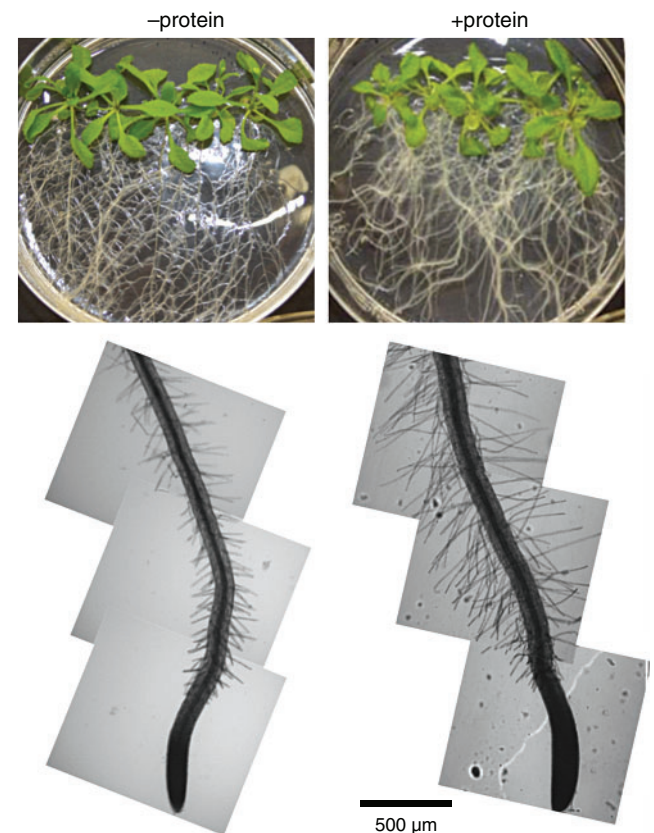

Fig. 4. Addition of protein to nutrient-replete axenic growth medium enhances the thickness of lateral roots and length of root hairs. Wild-type *Arabidopsis* plants (Columbia 0) were grown for 17 days on $0.5 \times$ MS medium (Murashige and Skoog 1962) with or without protein added (330 μg bovine serum albumen mL^{-1} growth medium; TGA Lonhienne, D Rentsch, T Näsholm, S Schmidt, C Paungfoo-Lonhienne, unpubl. data).

effect of inorganic nutrients on root morphology depends on the nutrient solute and its concentration. For example, supply of ammonium stimulates lateral root initiation and higher-order lateral root branching, while nitrate stimulates lateral root elongation (Forde and Walch-Liu 2009; Lima *et al.* 2010). Although there is a vast literature on the quantitative effects of inorganic nutrients on root growth and morphology (reviewed by Hermans *et al.* 2006), much less is known about the effects of organic forms on these characteristics. Amino acids and peptides alter root morphology and biomass, root length, thickness, surface area and root hairs (Walch-Liu *et al.* 2006*a*, 2006*b*; Cambui *et al.* 2011; Soper *et al.* 2011), providing indirect evidence that root structure and uptake of these compounds may be linked. Extension of the root system is particularly important for the acquisition of less mobile and less soluble nutrients such as most P-compounds (Bates and Lynch 1996). Root hairs have a large effect on the uptake of P-compounds as evidenced by morphologically different root hairs of crop species and varieties and their P-compound acquisition. Length and surface area of root hairs were significantly correlated with the depletion of P-compounds in the rhizosphere in low P growth condition (Bates and Lynch 1996; Gahoonia and Nielsen 1996; Gahoonia *et al.* 1997; Ma *et al.* 2001). Long root hairs are considered a beneficial trait for sustaining crop yields in low P soil (Gahoonia and Nielsen 2004). Although root hairs are under genetic control, their growth is modulated by environmental conditions. Increased root hair length under P-limitation is an adaptation that aids P-compound acquisition. Root colonisation with arbuscular mycorrhizal fungi is more pronounced in species and genotypes that have few or short root hairs (Smith and Read 2008). Root hair length increased in the presence of DNA in plants grown in P-replete medium (Paungfoo-Lonhienne *et al.* 2010*a*), pointing to a role of organic compounds as signalling agents. Similarly, phytate as a P source in axenic growth conditions increased root growth but dramatically decreased shoot growth (Richardson *et al.* 2000). The responsiveness of plants to organic compounds can be interpreted as a further indicator for their involvement in plant nutrient relations.
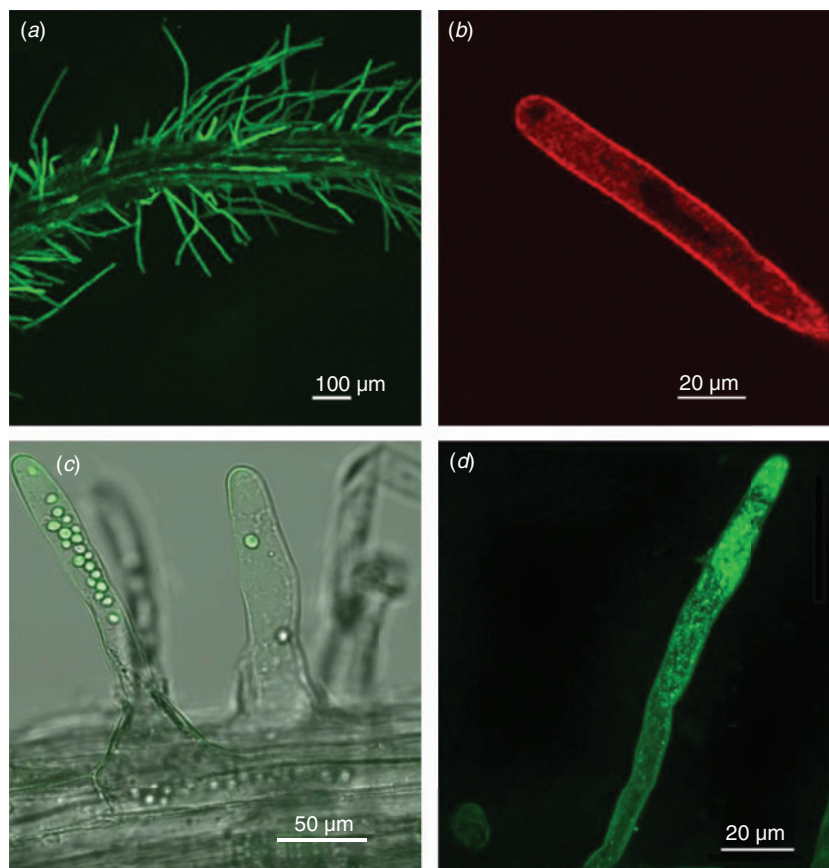
Fig. 5. Confocal laser scanning microscopy images of roots incubated with fluorescent protein, DNA and microbes. (*a*) Green fluorescent protein with *Arabidopsis* roots (Paungfoo-Lonhienne *et al.* 2008) (the copyright notice), (*b*) fluorescent-labelled DNA with *Arabidopsis* roots (Paungfoo-Lonhienne *et al.* 2010*a*) (www.plantphysiol.org, Copyright American Society of Plant Biologists, accessed 9 January 2013) and (*c*) yeast cells expressing GFP in roots of tomato (Paungfoo-Lonhienne *et al.* 2010*b*) (the copyright notice). (*d*) EGFP-tagged *Pseudomonas putida* PICP2 colonised olive root hairs (Mercado-Blanco and Prieto 2012) (reprinted from Plant and Soil, Vol. 361, p. 303, 2012 with permission of Springer).

Do root hairs provide a path for large molecule into roots?

Root hairs are specialised epidermal cells and the outermost interface between roots and soil. Root hairs can constitute up to 70% of the root surface. They are tip-growing extensions originating from root epidermal cells, so-called trichoblasts, which play an important role in water and nutrient uptake (reviewed by Datta *et al.* 2011). Root hairs are the major site of inorganic P uptake (Pi, phosphate) (Gahoonia and Nielsen 1998) containing the high affinity phosphate transporters responsible for P_i uptake (Mudge *et al.* 2002; Schunmann *et al.* 2004), and transporters for ammonium and nitrate (Lauter *et al.* 1996).

Tip growth in plant cells is an actin-based process that requires targeted exocytosis and compensatory endocytosis to occur at the growth cone and involves polarised membrane trafficking and the presence of endosomal compartments at the tip of root hairs (Ovečka *et al.* 2005; Voigt *et al.* 2005). Endocytosis occurs at the tip of growing root hairs and subsequent trafficking of the incorporated membrane. Mercado-Blanco and Prieto (2012) speculated that this process facilitates passive entry of bacteria into root hairs. Root hairs of N_2 fixing species facilitate the endophytic colonisation of roots, and recent studies indicate that root hairs of non-N_2 fixing angiosperm incorporate microbes (Fig. 5*d*; Prieto *et al.* 2011; Mercado-Blanco and Prieto 2012). Our research indicates that uptake of protein, DNA and microbes appears to be restricted to root-hair-producing trichoblasts (Paungfoo-Lonhienne *et al.* 2008, 2010*a*, 2010*b*) (Fig. 5*a*–*c*). These observations support the argument that root hairs are one of the entry routes of large molecule into root cells.

Conclusions

Mixotrophy is currently considered an exception in higher plants, and restricted to carnivorous, hemi-parasitic and partially hetero-mycotrophic species. However, similar to other mixotrophic organisms, notably phytoplankton, plants seem to be able to complement photosynthetic energy gain with essential elements from organic compounds and microbes. Although the contribution of organic carbon is likely to be less important for plants that are not light-limited, organic N and P compounds could supplement essential elements acquired as inorganic compounds. Knowledge of whether mixotrophy is important for plants only in certain ecosystems or whether it is a more general feature of plants is lacking. Similar to the uncertainty surrounding organic C fluxes in the rhizosphere (Jones *et al.* 2009), we have little quantitative information on how much organic versus inorganic N and P compounds contribute to plant nutrition. In semi-controlled growth conditions, several studies have shown a preference of plants for organic N monomers over inorganic N (e.g. Stoelken *et al.* 2010). Similarly, in highly artificial axenic growth conditions, *Arabidopsis* satisfied ~10% of N demand with protein (Paungfoo-Lonhienne *et al.* 2008). A knowledge gap remains in our understanding of how microbes, especially procaryotes, act in the rhizosphere, apoplast and as root endophytes. The rapid advances in microbial research will shed light into these questions, and more powerful analyses techniques will decipher the chemical nature and fluxes of organic compounds at much finer resolution than what has been possible in the past.

Approaches that span from molecular to ecological techniques, the use of mutants, sophisticated microscopy and modelling can be used to address the questions raised here. We argue that a new framework that considers plants as mixotrophs has numerous benefits including advancing the sustainable use of soils and efficient nutrient supply to plants with nutrients contained in organic materials. This is pertinent as the use of fertilisers is accelerating, much of which is applied as inorganic substances (or in the case of urea is rapidly converted to inorganics), and nutrient inefficiencies characterise many modern crop systems (Tilman *et al.* 2002; Davidson *et al.* 2012). Illustrating the dramatic discrepancy in fertiliser use is food production in China that increased 3.4-fold from 1961 to 2009, but was accompanied by 37- and 91-fold increases in N and P fertiliser application (Zhang *et al.* 2013). The resultant pollution is a problem of global significance (Gruber and Galloway 2008), motivating research and development on improving nutrient supply, uptake and assimilation by plants.

Acknowledgements

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Glossary

Autotroph: organism using light (phototroph) or electron donor (chemotroph) as energy and CO_2 as carbon sources, and inorganic compounds of all other required elements. In the text, 'autotroph' implies 'photoautotroph'

Auxotroph: organism which has a requirement for an external source of one or more vitamins

Heterotroph: organism without ability to photosynthesise (or undertake chemolitho-autotrophy) and using only organic carbon sources (plus anaplerotic rather than autotrophic CO_2 assimilation)

Kleptoplasty: retention of functional plastids from incorporated food sources by phagotroph algae

Mixotroph: autotrophic (photosynthesising) organism also acquiring organic sources of carbon (heterotrophy)

Obligate autotroph: an autotrophic organism which receives no nutritional benefit (in terms of growth rate) from any organic matter acquired by osmotrophy.

Osmotrophy: organism which takes up external solutes across the plasmalemma on a molecule by molecule basis.

Phagotroph: organism which engulfs smaller organisms as nutrient sources

Endocytosis: a process in eukaryotes and a planktomycete bacterium by which extracellular material such as macromolecules is incorporated into cells via a membrane trafficking system.

Phagocytosis ('cell eating'): endocytic processes that internalise particulate phases

Pinocytosis ('cell drinking'): endocytic processes that internalise fluid phases