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ORIGINAL PAPER

Effect of medium-pH and MES on adventitious root formation from stem disks of apple

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Abstract We have examined the effect of medium-pH on rooting using 1-mm slices cut from stems of apple microshoots. Before autoclaving, the pH of the rooting medium was set at various pH values between 4.5 and 8.0. During autoclaving, the pH drifted in particular in the alkaline region. Additional changes occurred during culture and the range set at 4.5–8.0 had shifted to 5.2–6.0 after autoclaving and 3 weeks of culture. When 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) had been added as buffering agent, the pH was stable when set at 5.0–6.5. Highest rooting was achieved at pH ~5.3 with and without MES (pH measured after autoclaving). This maximum did not correlate with highest auxin uptake. MES inhibited adventitious root formation during the initial phase of root formation when the meristemoids are being formed (ca. 30% reduction at 10 mM) but was promotive during outgrowth of the meristemoids to roots (30% increase at 10 mM). Inhibition and promotion by MES were not related to its buffering action as they were observed at all pHs.

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Abbreviations

IAA	Indoleacetic acid
IBA	Indolebutyric acid
MES	2-(*N*-morpholino)ethanesulfonic acid
MS	Murashige Skoog (1962)

Introduction

Plant tissue culture media are poorly buffered (Leifert et al. 1992; Vacin and Went 1949). From the various common medium components, only phosphate has buffering capacities, but the concentration of phosphate is low (1.25 mM in MS). Moreover, phosphate is rapidly taken up from the medium (Leifert et al. 1995). The pH of tissue culture media decreases among others by uptake of NH_3^+, and increases by uptake of NO_3^- (e.g. Schmitz and Lorz 1990). A change of medium-pH may have various effects that may influence performance and development of the explants (George et al. 2008). (1) In semi-solid media, the availability of many compounds is pH-dependent (Arnon et al. 1942; Scholten and Pierik 1998; Van Winkle et al. 2003). (2) Uptake of medium components by the explants may be influenced directly by the pH. This holds in particular for uptake of auxins (see Discussion). (3) The pH influences enzymatic and other chemical reactions in

the medium. In this context, it is important to note that in media, especially near explants, enzymes and other compounds occur released from explants either because of wounding at subculturing or during culture by excretion/secretion. (4) Finally, low pH prevents agar from solidifying.

It has been reported that medium-pH influences developmental processes in tissue culture, among others regenerative processes: xylogenesis in *Citrus* and *Zinnia elegans* (Khan et al. 1986; Roberts and Haigler 1994); androgenesis in winter triticale and wheat (Karsai et al. 1994), adventitious bud regeneration in tobacco (Pasqua et al. 2002) and adventitious root formation in apple (Harbage et al. 1998). For xylogenesis in *Z. elegans*, it has been reported that the effect of pH correlates with changes in uptake of auxin (Shinohara et al. 2006).

In this paper, we report the effect of pH and the buffer MES on rooting of 1-mm thick slices cut from stems of apple microcuttings. These explants are very suitable to study the effect of pH on rooting because they are tiny, thereby minimizing the role of the explant itself. As auxin is a key factor in rooting, the effects of pH on IAA uptake and decarboxylative oxidation have been examined.

Materials and methods

Apple shoot cultures

Shoot production of *Malus* 'Jork 9' was maintained as described previously (De Klerk et al. 1995). Shoots of 1 cm were cultured in tubes with 15 ml propagation medium. After 5 weeks at 25°C and a 16-h photoperiod (35 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps), clusters consisting of 5–10 shoots had been formed by axillary branching. Shoots were excised and either used for further shoot proliferation or for preparing 1-mm slices.

Preparation and culture of 1-mm slices

The leaves were removed from the stems and 1-mm slices were cut with a device consisting of 11 razor blades separated by 1-mm metal plates. Slices cut from the middle 1 cm of stems, show no effect of the position of the slice in the stem (apical, middle, basal) (de Klerk and Caillat 1994). The slices were cultured with the apical side down on a nylon mesh on top of 30 ml of rooting medium in a 9-cm Petri dish (De Klerk et al. 1995). The Petri dish was incubated upside down in the dark in a culture room at 25°C. The concentrations and type of auxin are indicated for each experiment. The medium was solidified with 0.7% agar (BBL granulated) with the exception of the experiment with labelled IAA in which 0.2% gelrite was used to allow determinations at low pH (Fig. 5). After 5 days of culture in the dark, the slices were transferred to hormone-free medium and to the light. For this transfer, the nylon mesh with the slices attached was transferred. Excess liquid was removed by placing the mesh on filter paper (5 s). In each Petri dish, 30 slices originating from six shoots were cultured. The five slices taken from each shoot were nonadjacent because it has been shown previously there exists a (weak) correlation between the capabilities to root of adjacent slices; there is no correlation between the rooting of nonadjacent slices (De Klerk and Caillat 1994). Roots were counted after 21 days under a dissecting microscope. MES was added as indicated for each experiment. The pH was set before autoclaving and varied as indicated for each experiment. The pH was set and measured with a conventional pH meter.

Uptake and oxidation of 1-^{14}C-IAA

The experiments were performed in 2-cm high 9-cm Petri dishes. The dishes contained 20 ml rooting medium (De Klerk et al. 1995) with 10 μM IAA. One kBq [1-^{14}C]IAA (Sigma, 1.48 10^6 kBq $mmol^{-1}$) was added as a tracer. Both uptake of the label by explants and $^{14}CO_2$ (formed by oxidation of [1-^{14}C]IAA) in the headspace of the Petri dish were measured. For uptake, samples of three slices were taken at random and digested in 200 μl Soluene-350 (Packard) overnight at 40°C. Then 4.5 ml Hionic-Fluor (Packard) was added, and $^{14}CO_2$ was trapped in 300 μl 2 M KOH in a small vial with a piece of filter paper. To determine the amount of radioactivity, 4.5 ml Ultima Gold (Packard) was added to the 300 μl KOH solution and the filter paper.

Statistics

For the determination of root numbers, three Petri dishes with 30 slices each were used for each

treatment and for the experiments with [1-^{14}C]IAA, two Petri dishes with 30 slices each. Uptake of the label was determined by taking two samples of three slices from each dish (four samples in total). The data on $^{14}CO_2$ are means of two Petri dishes. To establish the significance of the pH-effect in Fig. 5, we calculated whether the slope of the regression line was significantly different from zero.

Results

Shift of pH during autoclaving and culture and the effect of MES

Without addition of MES, the agar did not solidify when the pH had been set at 4.0 or lower, so the lowest pH looked at was 4.5. A large shift of pH occurred after autoclaving, especially when the pH had been set higher than 6.0 or higher (Fig. 1). In all cases, the pH dropped: the initial range of 4.5–8.0 changed to 4.4–5.5. During culture, the pH continued to change in medium without MES and always increased relative to the pH just after autoclaving. The range 4.4–5.5 (measured after autoclaving) changed to 5.2–5.8 after 21 days of culture (Fig. 1). When the pH had initially been set at 5.5 (close to the pH at which tissue culture media are usually set), the changes were small.

With addition of 10 mM MES, the agar did not solidify at pH 4.5. With 10 mM MES, after autoclaving a large pH shift occurred only at pH $>$ 6.5. This is beyond the buffering capacity of MES (Good et al. 1966). There was no or only a minor additional change during culture (Fig. 1). MES is the buffer of choice for tissue culture media because it buffers in the appropriate range (pH 5.5–6.7) and supposedly has little or no effects unrelated to buffering (Banthorpe and Brown 1990; Good et al. 1966; Parfitt et al. 1988). In preliminary experiments, though, we observed an inhibitory effect of MES on rooting. A dose–response curve confirmed the inhibitory effect (Fig. 2). Therefore, a low MES concentration of 10 mM was taken for the experiments.

In the experiment shown in Fig. 2, MES had been added during the initial 5 days only. During this period, three distinct steps may be distinguished on basis of their hormonal requirements: (1) dedifferentiation (0–24 h), (2) induction (24–96 h), and (3) differentiation (from 96 h onwards) (see for explanation De Klerk et al. 1995). We examined whether the inhibition by MES is similar in these phases by

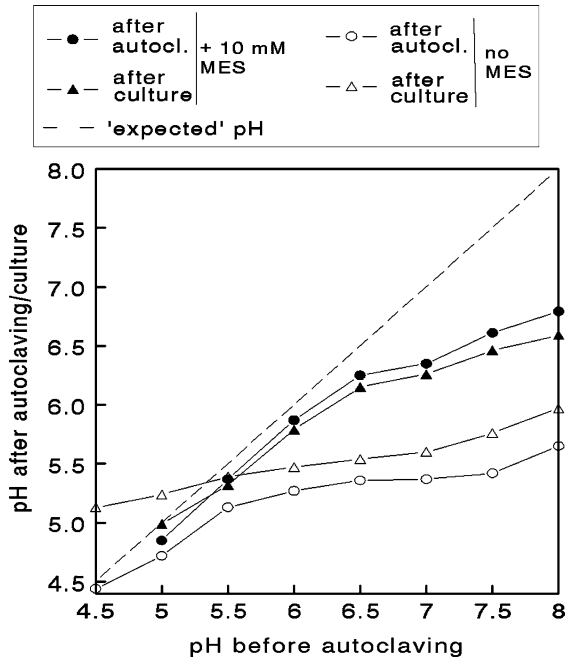

Fig. 1 Effects of autoclaving and 3 weeks of culture on pH of nutrient medium with or without 10 mM MES

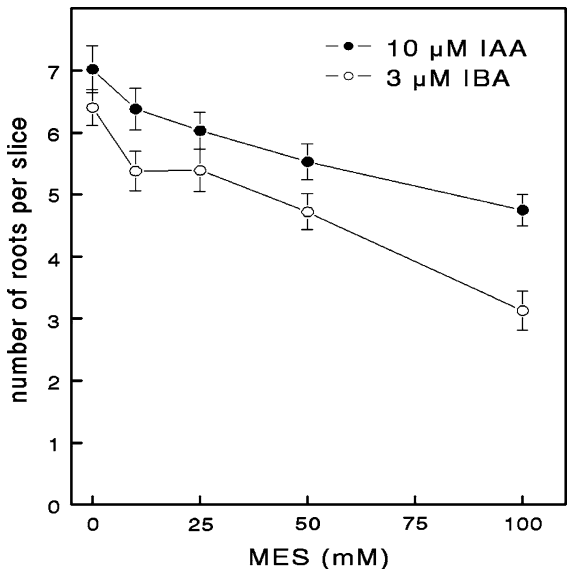

Fig. 2 Effect of MES (pH 5.5) on adventitious root formation from 1-mm thick stem slices excised from microcuttings of *Malus* 'Jork 9'. MES and auxins were applied for the initial 5 days only. After that the slices were transferred to hormone-free medium without MES

giving 24 h-pulses with MES. Figure 3 shows that inhibition strongly depended on the timing of the pulse with highest inhibition during pulses at the 3rd and 4th day, so during step 2, the induction phase.

The effect of pH on rooting

To examine the effect of pH, slices were cultured the initial 5 days at various pHs with or without 10 mM MES and after that transferred to standard medium. During this initial period, the root meristems are being formed (Jásik and De Klerk 1997). In Fig. 4a, the number of regenerated roots is plotted as a function of the pH measured at the time of transfer of slices to the Petri dishes (the pH just after autoclaving). The pH had a significant effect on the number of adventitious roots. Highest rooting was at pH ~5.3 both with and without addition of MES. MES inhibited rooting by 30–50%. The shape of the pH response curves with and without MES were similar, indicating that the inhibition by MES was not related to its buffering action.

We also examined the effect of the pH on outgrowth of the meristems to roots. For this experiment, the slices were cultured 5 days on rooting medium with an optimal concentration of IBA (3 μM) at standard pH without MES and then transferred to auxin-free media at a range of pHs with or without MES. Again the numbers of roots are plotted as a function of the pH measured at the time of transfer. Figure 4b shows that there is no effect of pH. Interestingly, during this stage MES has a promotive effect ($P < 0.001$). This stimulating effect occurred at all pHs tested, indicating again that its effect was unrelated to its buffering action.

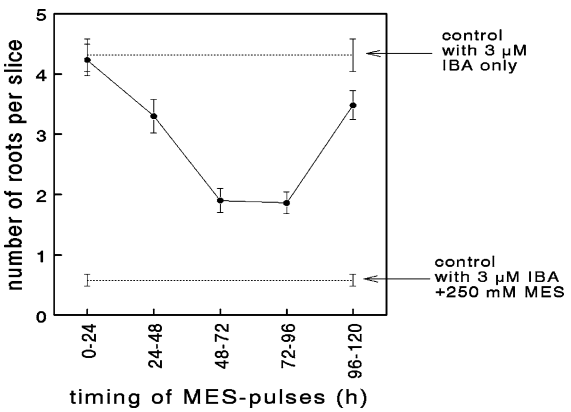

Fig. 3 Effect of 24-h pulses with 250 mM MES (pH 5.5) on rooting of 1-mm stem slices excised from microcuttings of *Malus* 'Jork 9'. Slices were cultured for 5 days on medium containing 3 μM IBA and the pulses with MES were given at the indicated periods. During the MES-pulses, 3 μM IBA was also present in the medium. After the 5-days period, the slices were transferred to hormone-free medium and to the light. A control with 3 μM IBA only and a second control with 3 μM IBA + 250 mM MES were also carried out. In these controls, no 24 h transfers were made. It has been found previously that 24 h transfer to fresh medium does not influence rooting. The numbers of roots were counted after 21 days

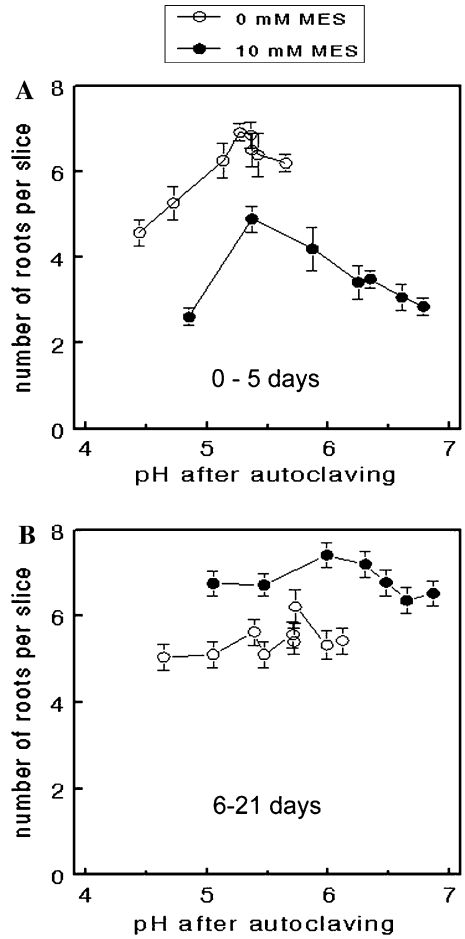

Fig. 4 Effect of pH on adventitious root formation from 1-mm thick stem slices excised from microcuttings of *Malus* 'Jork 9'. The pH at the x-axis is the pH measured after autoclaving. The pH was either the pH of the standard rooting medium (0 mM MES) or rooting medium with 10 mM MES. In (**a**) the effect of pH during the initial 5 days was determined with 3 μM IAA. After that, the slices were transferred to standard medium without auxin and without MES. In (**b**) the effect of the pH in the following 6–21 days was determined: the slices were cultured with 3 μM IBA and after 5 days transferred to hormone-free medium with increasing pHs

Effect of pH on IAA uptake and oxidation

Increased rooting may be related to increased uptake of auxin. Therefore we examined uptake of auxin at a range of pHs. As IAA is massively decarboxylated (Guan and De Klerk 2000), we also examined the effect of pH on oxidation. When slices were cultured at a range of pHs, both uptake and oxidation of IAA increased with decreasing pH (for all: $P < 0.002$). In this experiment, the pH was kept stable by 10 mM MES. The experiment was carried out with gelrite to get solid medium at low pH. In the range that has been studied (4.5–7) both uptake and oxidation were highest at the lowest pH. A decrease of the pH from 7 to 4.5 doubled both oxidation and uptake (Fig. 5).

With respect to the pH-effect on oxidation, the "site of action" is not known: Oxidation may occur both within and outside the tissue and at the interphase. Since apple stem explants excrete peroxidases (P. Huisman and G. J. de Klerk unpublished results) and peroxidases oxidize auxins (Gazaryan et al. 1999), oxidation of IAA in the medium is likely. We examined this with slices that had first been preincubated for 24 h. In this way, enzymes released from damaged cells, were avoided. The preincubated slices were incubated for 24 h on medium with labelled IAA and then removed. Oxidation of IAA was determined both during the 24 h culture period and during the following 24 h in the absence of slices. Since photooxidation was avoided by culture in the dark, measured oxidation has been brought about by compounds released into the medium from the slices. Significant oxidation occurred during the 24 h period without slices (Table 1). Similar results were obtained when the slices had been preincubated for 48 h.

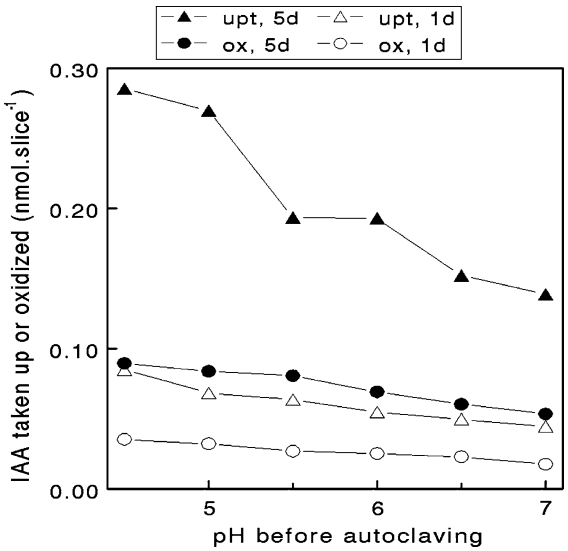

Fig. 5 Effect of pH on uptake and oxidation of IAA after 1 day of culture on medium with 10 μM IAA and a tracer amount of ^{14}C-IAA. The medium was solidified with 0.2% gelrite to enable experimentation at low pH. Uptake and oxidation were measured after 1 and 5 days

Discussion

Stability of pH and the morphogenetic effect of MES

To evaluate the effect of pH, the actual pH in the medium is important. Various studies have shown that the pH of tissue culture media is poorly-controlled and shifts both during autoclaving and during culture (e.g. Skirvin et al. 1986; Vacin and Went 1949). We obtained similar results (Fig. 1). Thus we measured the pH after autoclaving and plotted this pH in the graphs in Fig. 4. It should be noted that both during autoclaving and during culture, the change at pH 5.5 (this is close to the pH commonly used in tissue culture) was only slight (Fig. 1). When MES buffer had been added to maintain the pH, the shift in the range 5.0–6.5 was reduced to ca. 0.2 pH units.

MES is considered to be an inert buffer but toxic at high concentration (>10 mM; Parfitt et al. 1988). An interesting finding of this paper is the inhibition by MES during the initial 5 days of the rooting treatment (Fig. 4a) and the promotive effect during the outgrowth of meristemoids to roots (Fig. 4b). As both promotion and inhibition occurred at all pHs, they were not related to the buffering action of MES. The timing of inhibition by MES was established more precisely by giving 24 h pulses with MES. We found stage-specific inhibition during the induction phase, i.e. the period during which the root meristemoids are being formed by the rhizogenic action of auxin (De Klerk et al. 1995). The promotion of outgrowth of primordia and the stage-specific inhibition show that the effect of MES is not caused by toxicity. The morphogenetic effect of MES corresponds with the effect of an anti-auxin. There are several reports in

Table 1 Oxidation of IAA

	Oxidation during 24 h in the presence of slices (nmol IAA slice^{-1})	Oxidation during the next 24 h in the absence of slices (nmol IAA slice^{-1})
24 h preincubation	0.083	0.028
48 h preincubation	0.071	0.028

Slices were preincubated for 24 h or 48 h on medium without carboxyl labelled IAA, then transferred for 24 h to medium to which a tracer amount of ^{14}C-IAA had been added and after that transferred back to the previous medium. Oxidation of ^{14}C-IAA was determined in the Petri dishes with labelled medium both during the presence of slices and in the 24 h period after that (when no slices were in the dish). The dishes always contained 10 μM IAA and were kept in the dark to avoid photooxidation

which biological effects of low concentrations of MES (10 mM or less) have been reported. In peach embryo culture, MES decreased embryo survival (Sinclair and Byrne 2003). In *Medicago* species, MES promoted nodulation (Ewing and Robson 1991). In *Hyoscyamus niger*, the formation of morphological abnormal somatic embryos was observed at 10 mM MES (Tu et al. 1996). Baker et al. (2007) report that MES at concentrations of 5 mM and higher can interfere with the oxidation of certain phenolics. It is interesting to note that an other biological buffer, piperazine, has a major stimulating effect on adventitious root formation (Liu et al. 1995).

The effect of pH on rooting

The pH influenced rooting during the first 5 days when the root meristems are being formed whereas after that during the period in which the meristemoids develop into roots, the pH had virtually no effect. For assessment of the mechanisms underlying the pH-effect, it is important to discriminate between the various locations where the pH might have an effect: (1) the explant, (2) the medium and (3) the interface between explant and medium.

Within the cells in the explants, the various compartments have different pH values and the pH is maintained (Felle 2001). In the symplasm, the pH of the cytoplasm is ~7 and of the vacuole ~5. The apoplasm has a pH of ~5. The maintenance of the pH is illustrated in an experiment with detached leaves of *Vicia faba* (Felle and Hanstein 2002). When the leaves were placed in a 10 mM MES–TRIS buffer and the pH was changed, the changes in pH in the apoplast were small: With an initial buffer pH = 4.1 and transfer to buffer pH = 6.8, the apoplastic pH of substomatal cavities increased from 4.68 to 5.14 and in the reverse transfer decreased from 5.16 to 4.70. This indicates that the pH of the apoplasm is not strongly influenced by the medium but stays close to the 'natural' pH of ca. 5.0. The symplasm has a much larger capacity to buffer (Felle 2001) so the pH of cytoplasm and vacuole will be even less influenced by the medium-pH. So, within the explant the pH of both apoplast and symplast will be hardly affected by the medium-pH, in particular when no buffering agent has been added. This probably also applies to the site in the apple stem disks from where the roots are regenerated which is a few cell-layers from the cut surface (Jásik and De Klerk 1997). It should also be noted that we observed the effect of the pH both with and without MES (Fig. 3a). In conclusion, the site of action of the medium-pH is most probably the medium and/or the interface between explant and medium.

The most important candidate to explain the pH-effect is auxin uptake. Depending on the pH and their pKa, auxins are either present as undissociated molecule or as anion. In the apoplast, the pH is low (ca. 5) so a large percentage of auxin is present as undissociated molecules that may pass through the membrane by diffusion. The anion may be taken up by a carrier (Delbarre et al. 1996; Morris 2000). In the cytoplasm (ca. pH 7), most auxin is present as anion and cannot diffuse out. So, auxin uptake expectedly increases with a decrease of the pH. Figure 5 shows that indeed uptake increases with a decrease of the pH.

The pH may also influence (enzymic) reactions. The explant excretes peroxidases in the medium and some peroxidases oxidize IAA. Peroxidases have an

acidic pH optimum (Zmrhal and Machackova 1978). Indeed, we found a pH-effect corresponding to an acidic pH optimum (Fig. 5). Thus, at low pH less label would occur in the slices because of increased oxidation. As we found the opposite, more label at low pH, the effect op low pH on uptake is more significant than appears at first sight from Fig. 5.

The optimal pH during the initial 5 days was ~5.3 (Fig. 4a). At pH higher than 5.3, both auxin uptake and rooting decreased (Fig. 5). So in this case, decreased rooting may have been brought about by decreased auxin uptake. At pH lower than 5.3, auxin uptake increased (Fig. 5) and rooting decreased. Obviously, the decrease in rooting cannot be attributed to altered auxin uptake. It should be noted here that we actually did not determine auxin in the uptake experiments, but label after adding labelled IAA (Fig. 5). After uptake, auxin is massively conjugated and as a result inactivated. Harbage et al. (1998) did find an effect of pH on auxin uptake but not on auxin conjugation. Therefore, the amount of label is probably an accurate indication of the amount of IAA in the tissue. The decrease of rooting at pH < 5.3 may be explained altered uptake of specific medium compounds, among others inorganic nutrients (cf. Pasqua et al. 2002).

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