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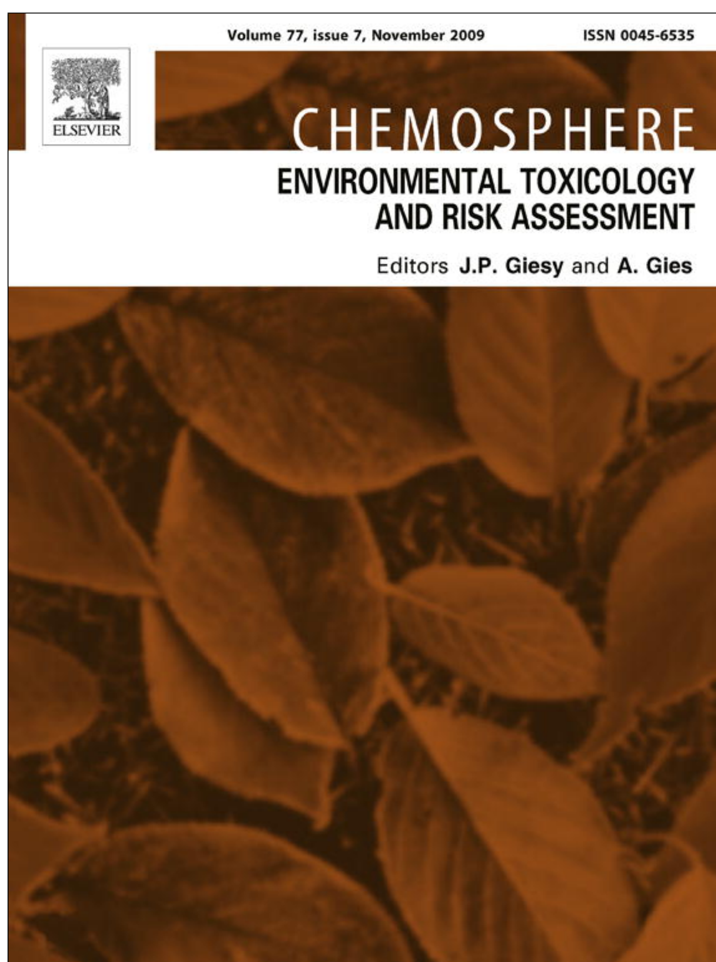

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Development of a naturally miniaturised testing method based on the mitochondrial activity of fern spores: A new higher plant bioassay

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ABSTRACT

One of the main concerns of current environmental toxicology is the low number of taxa used for standard bioassays. Ferns, with more than 10 000 living species, are the second largest group of vascular plants and are important components of numerous plant communities. Fern spores and gametophytes have long been recognized as useful models for plant research since they constitute a naturally miniaturised and economic higher plant model. Mitochondria are the main energy source in eukaryotic cells and any toxic damage will affect the whole organism. The reduction of tetrazolium salts to water-insoluble coloured formazan salts by the respiratory chain has been used for more than 50 years as a measure of cell mitochondrial activity and viability in eukaryotic organisms. Here, the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) by mitochondria is adapted and optimized to measure fern spore or gametophyte viability. Procedures selected as optimum in the model species *Dryopteris guanchica* are as follows: bleach sterilization, incubation without shaking at 20 °C in the dark for 1–4 h with 0.05–1.5% TTC in Dyer medium supplemented with 0.001–0.005% Tween 20 at pH 8. We conclude that this method constitutes a promising low cost bioassay for higher plant toxicity during development.

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1. Introduction

One of the main concerns of current environmental toxicology is the low number of taxa used for standard bioassays. The use of an adequate range of taxa in ecotoxicological studies is a key point for the achievement of ecologically relevant results. Higher plants are an essential part of a healthy and balanced ecosystem and new plant models are essential in the evaluation of potential impacts of chemicals on non-target species (Gong et al., 2001).

Ferns are important components of numerous plant communities. With more than 10 000 living species, ferns are the second largest group of vascular plants (Smith, 1972 in Schneider et al., 2004). Fern spores, and spore-developed gametophytes, have long been recognized as useful models for plant research in important areas, namely: plant development, sex determination, gamete production and fertilization, response to environmental factors and evolution of plant complex traits (Banks, 1999). One of the main advantages of this model is its naturally miniature size. Fern spores are single meiotic cells which develop into gametophytes that are miniature mature higher plants. In this case, the use of microtubes and microplates is imposed by the natural model. The development of rapid and reliable methods to test phytotoxicity with fern spores and gametophytes could dramatically reduce standard tests costs maintaining the biological relevance of whole plant testing.

Here we propose a fast method for the study of fern spore and gametophyte viability: the measurement of mitochondrial activity. Cell mitochondrial activity has been used for years as a viability assay in eukaryotic organisms since the reduction rates of the respiratory chain reflect the respiratory activity of cells. Any toxicant affecting mitochondrial activity and cell energy budget will alter normal plant development, even jeopardising plant survival. The most used assay is the reduction of tetrazolium salts to water-insoluble coloured formazan salts by the respiratory chain (Kalina and Gahan, 1968; Altman, 1976). The reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to the corresponding triphenyl formazan salt (TPF) has successfully been used in the analysis of the viability of seeds or vegetal cells since 1951 (Smith, 1951; Kalina and Palmer, 1968) but has not been applied to fern spores or gametophytes yet.

The adaptation of TTC method to fern spores and gametophytes presents some problems. The presence of highly hydrophobic substances in the cellular wall makes the spores difficult to suspend in aqueous media. Besides, fern spore cellular wall is extremely resistant and therefore the methods used for sample homogenization and TTC extraction must be especially vigorous. On the other hand, most existing methods use ethanol 95% to extract TPF, however,

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ethanol used to extract TPF also extracts various pigments from plant tissues (i.e. chlorophyll) and can interfere in the spectrophotometric measurement.

The aim of this work is the development of a new higher plant bioassay based on the tetrazolium salts reduction to coloured formazan salts by the mitochondrial activity of fern spores.

2. Materials and methods

2.1. Plant material

Dryopteris guanchica Gibby and Jermy spores were sampled in NW Spain, A Coruña province, San Xusto river. Fragments of leaf were collected with mature but closed sporangia. Spore release was promoted by drying the fragments on smooth paper for a week in the laboratory. Spores were stored dry at 4 °C in darkness until use.

2.2. Preparation of sterile suspensions of fern spores

Spores were sieved through porous paper (Whatman paper grade 105, Whatman plc, Kent, England) to eliminate rests of sporangia and weighed on a Petri dish, then suspended in gametophyte culture medium (Dyer, 1979) supplemented with Tween 20, 0.001–0.005%, a detergent used as wetting agent, (Dyer–Tween medium) and hydrated during 24 h at 20 °C (PAR 35 μmol m^{-2} s^{-1}, 16 h photoperiod).

After imbibition, spores were transferred to 2 mL conical microtubes and sedimented by centrifugation. All centrifugations were carried in a Heraeus Biofuge Primo (Thermo Scientific, Waltham, MA, USA) for 5 min at 600*g*. Supernatant was discarded and 1800 μL of commercial bleach 1:100 added. After 5 min of soft agitation spores were sedimented, bleach was discarded and pellet was washed twice in excess of Dyer–Tween medium to eliminate bleach by dilution of several orders of magnitude. After the last centrifugation, pellet was resuspended in a known volume of Dyer–Tween and the concentration of the spores was calculated with the aid of a Fuchs–Rosenthal calibrated chamber. The mean of at least three independent recounts was used as final cell concentration.

A germination assay was conducted to assess the effect of sterilization on spore viability as previously reported elsewhere. Briefly, spores of bleach-treated suspensions and untreated spores were sown on culture medium solidified with agar in 5.5 cm diameter Petri dishes. Spores were incubated in a growth chamber (20 °C, PAR 35 μmol m^{-2} s^{-1}, 16 h photoperiod). For each treatment, four Petri dishes were incubated. After 1 month 100 spores on each dish were randomly selected and germination was determined.

2.3. Tetrazolium assay

The particular assay conditions used are given in each figure legend. In general TTC (Sigma, St. Louis, MO, USA) was dissolved in Dyer–Tween culture medium and pH was set to 8.0. The autocleaved TTC solution was added in excess to the cells and incubated in 2 mL conical microtubes without shaking for 4 h in the dark at 20 °C. After incubation, spores were sedimented by centrifugation (5 min, 600*g*) and washed twice with fresh culture medium. Washing steps guarantee elimination of contaminating TTC by a dilution of several orders of magnitude.

Due to the characteristics of fern spore wall, several sequential steps combining chemical, thermal and mechanical homogenization methods were necessary for an optimal extraction of the red formazan salt. First, a mixture consisting of 240 μL KOH 0.1 N and 240 μL ethanol 96% (*v*/*v*) was added to the spore pellet and sonicated in an Selecta Ultrasons bath during 1 h (40 kHz, 100 W) at room temperature, then samples were incubated at 65 °C during 15 min in a water bath. Tubes were cooled at room temperature during 10 min and samples were grinded in glass conical homogenizers during 1 min at constant speed.

2.4. Partition of TPF from water-soluble plant pigments

TPF was extracted in 1000 μL of *n*-hexane by strong agitation (3000 rpm) during 10 min with a vortex Vx100 (Labnet, Edison, NJ, USA). Phase separation was performed manually with Pasteur pipettes after 7 min centrifugation at 1100*g* in a Heraeus Fresco Biofuge (Thermo Scientific, Waltham, MA, USA). The absorbance of the red TPF salt dissolved in *n*-hexane was read at 492 nm in a Spectronic Genesys 8 UV/Vis spectrophotometer.

2.5. Bioassay

A toxicity range-finding test consisting of an abbreviated acute test using a logarithmic series of toxicant dilutions was performed, as recommended by US Environmental Protection Agency. We used a mixture of the polycyclic aromatic hydrocarbons (PAH) phenantrene, anthracene and naphthalene at the same concentration, and an organic extract of an industrial contaminated soil with unknown composition.

Spores were sieved through porous paper to eliminate rests of sporangia and weighed on a Petri dish, then suspended in Dyer–Tween medium as explained above. Spores were then sterilized with bleach and counted. Aliquots containing 2.5×10^4 spores were then prepared and 1 mL of Dyer–Tween medium containing different concentration of pollutants was added. The spores were incubated during 24 h (20 °C, PAR 35 μmol m^{-2} s^{-1}, 16 h photoperiod) and then the TTC assay was performed with TTC 0.6%, pH 8 during 1 h in darkness.

3. Results

A germination assay showed that *D. guanchica* spores were not damaged by the use of Tween 20, bleach or centrifugation during the preparation of sterile suspensions of fern spores (Material and methods). All the dishes sown with sterilized spores showed more than 95% germination success, similar to untreated spores (data not shown). Current assays with other non chlorophyllic species are yielding similar results; however, we have stated in *Osmunda regalis* green spores that these treatments produce alterations in respiration rates and the lyses of the spore.

The absorption maximum of TPF in *n*-hexane is at 492 nm, therefore all the absorptions reported here were measured at 492 nm. We observed that TPF in *n*-hexane is photosensitive and turns into a yellowish substance, especially if maintained cold (i.e. on ice), so samples were protected from light at room temperature and absorbance measured immediately after extraction.

The effect of the pH of the incubation medium on the amount of TPF salts produced by *D. guanchica* spores was assayed. The optimum pH for TPF production is 8.0 although there are no significant differences between pH 5 and 10 (data not shown). A time course study of colour production at 20 °C shows that the maximum production of TPF is at 4 h incubation (Fig. 1). Afterwards there is a strong decrease in colour formation that may reflect a viability loss due to end product degradation. Therefore, all subsequent experiments were performed at pH 8.0, 4 h incubation at 20 °C, in the absence of light and without agitation.

There is a direct relation between TTC concentration in the incubation medium and colour formation up to 1.5% and no toxic

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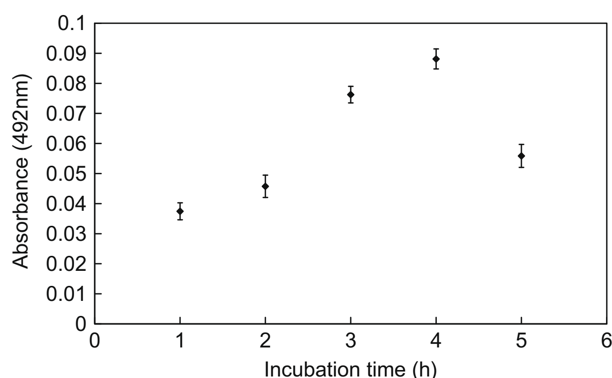

Fig. 1. Amount of formazan salt produced at different time periods. The samples were incubated with 0.3% TTC pH 8, in darkness at 20 °C. Values are means ± SD of eight replicates with 5×10^4 spores per replicate.

effects are observed in the conditions studied (Fig. 2). In the following assays we chose 0.3% TTC as working concentration for economy reasons but higher concentrations may be used. There is a slight residual colour at TTC 0% due to spore pigment absorbing at 492 nm that is extracted together with TPF.

In order to get a better insight of the contribution of pigments together with non enzymatic TTC reduction, an assay with spores killed in Pasteur oven (dry heat, 100 °C, 18 h) and TTC 0.3% was performed. The residual absorbance due to cell pigments increases with the number of cells (Table 1) and may vary with the species studied (data not shown).

Fern spores possess a thick wall that isolates and protects them from environmental physical or chemical stress. We destructed the lipid membranes and wall structural components of the spores with 0.1 N KOH:EtOH 96%, 1:1, *v*/*v*, aided by 1 h sonication in bath

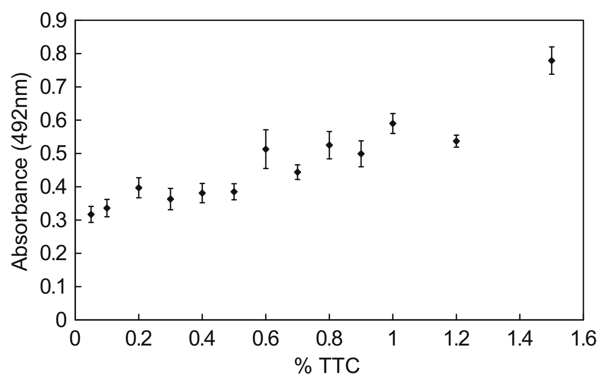

Fig. 2. Effect of concentration in the TTC enzymatic reduction. Spores were incubated in darkness, at 20 °C during 4 h, and various % TTC pH 8. Each value is the mean ± SD of nine replicates in three independent assays with 1×10^5 spores per replicate.

Table 1
Residual absorbance (mean ± SD) of killed spores. Cells were killed in a Pasteur dry oven at 100 °C during 18 h. Different numbers were incubated during 4 h with 0.3% TTC pH 8 in darkness at 20 °C.

Number of spores	Absorbance (492 nm)
0	0
2.00×10^4	0.005 ± 0.001
4.00×10^4	0.016 ± 0.004
6.00×10^4	0.031 ± 0.002
8.00×10^4	0.048 ± 0.001
1.00×10^5	0.051 ± 0.001

followed by 15 min incubation at 65 °C and mechanical homogenization with a glass conical tissue grinder. However, we observed that tissue grinding must be performed after sonication and thermal treatment or it can even worsen extraction yield (Fig. 3).

n-Hexane extraction of the sample with a volume ratio of 2:1 is performed during 10 min with strong vortex agitation. TPF recovery with two subsequent *n*-hexane extractions resulted in its contamination with unwanted substances that strongly disturbed absorbance at 492 nm. Although the phase separation is very rapid, samples were centrifuged (7 min, 1100*g*) in order to sediment possible contaminants out of the interphase. The extraction of TPF with DMSO produced high levels of pigment contamination (results not shown).

In order to use mitochondrial activity test as a viability assay, the reduction by the respiratory chain of TTC must quantitatively reflect the respiratory activity of cells. We observed that the amount of TTC reduced is highly correlated with cell number (Fig. 4).

Preliminary toxicological studies showed that *D. guanchica* spores undergo an inhibition of mitochondrial activity in the presence of a mixture of aromatic compounds (anthracene, phenanthrene and naphthalene) in concentration as low as 0.0001% in Dyer–Tween medium. We obtained similar but less intense results using an extract of an industrial contaminated soil (Fig. 5).

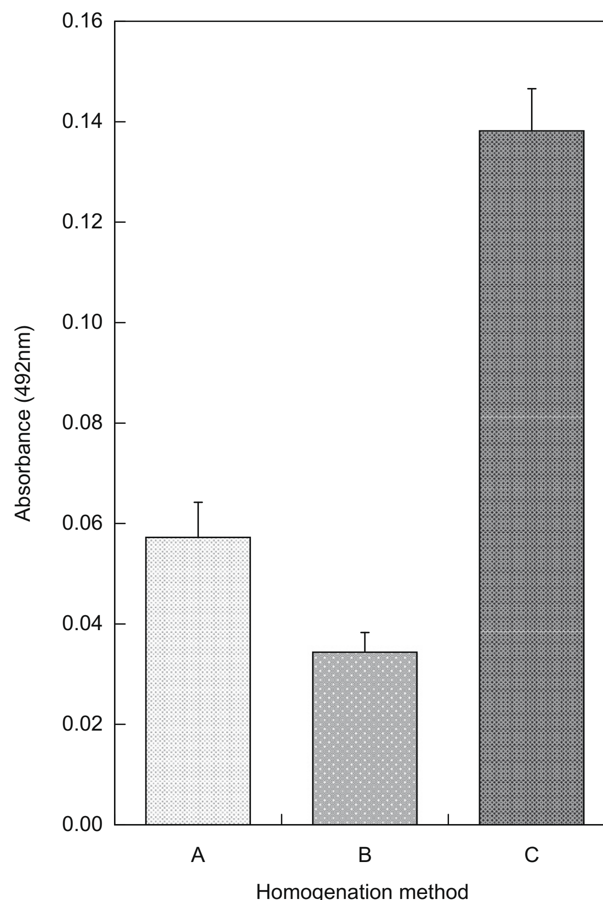

Fig. 3. Effect of the homogenization method in the solubilization and recovery of mitochondrial formazan salts. To extract the formazan salts formed during spore incubation with TTC a mixture of KOH/EtOH was added to all the samples, then they were sonicated during 60 min and incubated at 65 °C in a water bath during 15 min. The bars show: (A) no tissue grinder used; (B) tissue ground in a glass conical grinder before thermal treatment; (C) tissue ground in a glass conical grinder after thermal treatment. The data presented is the mean ± SD of three independent experiments with at least nine replicates per value.

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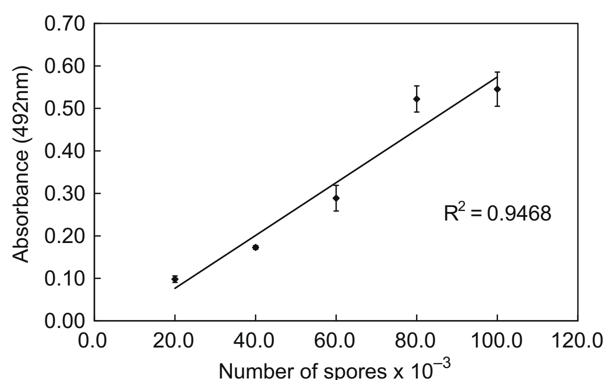

Fig. 4. Amount of formazan salts produced by different numbers of *D. guanchica* spores. Cells were incubated during 4 h with 0.3% TTC pH 8 in darkness at 20 °C. The results presented are the mean ± SD of three independent assays and eight replicates.

4. Discussion

Ferns, with more than 10 000 living species, are the second largest group of vascular plants and are important components of numerous plant communities. Therefore, the inclusion of this taxon into the standard bioassays for ecotoxicological assessment is an important goal. Besides, the miniature size of fern spores and gametophytes allows the use of high throughput technology which dramatically reduces testing costs.

TTC reduction has been used as a mitochondrial activity assay for many years and represents a simple, rapid and inexpensive means of assessing viability when dealing with large numbers of samples (Towill and Mazur, 1975; De Block and De Brouwer, 2002).

This paper presents a low cost viability assay for fern spores based on the reduction of TTC which can be the basis for a new bioassay. *D. guanchica* has been chosen as model species for the study presented. This evergreen fern is located in some Canary Islands and in northwestern Iberian Peninsula. Although several species have been tested (*Dryopteris aemula, D. affinis, D. corleyi, D. filix-mas, D. oreades, O. regalis* and *Polystichum setiferum*), *D. guanchica* was the most suitable because it is the only species that produces spores throughout the year. This trait has guaranteed the relatively high amounts of spores needed to optimize a new method. *P. setiferum* also showed good results which have not been shown here. The stabilization of fern spores and gametophytes in aqueous suspension is difficult given their natural hydrophobicity, so the addition of a wetting agent, the detergent Tween 20, and a special care in homogenizating the suspension before pipetting is necessary. Bacteria and fungi are also able to reduce TTC salts so a sterilization step with 1:100 commercial bleach:water must be performed before TTC incubation.

Standard procedures selected as optimum in the model species *D. guanchica* are as follows: bleach sterilization, incubation without shaking at 20 °C in the dark for 1–4 h with 0.05–1.5% TTC in Dyer medium supplemented with 0.001–0.005% Tween 20 at pH 8.

Given the ample distribution of ferns, spores from native species can be used in order to adapt the testing conditions to geographic regions and habitats. We recommend performing a preliminary study for other species in order to find the specific optimum conditions for sterilization, incubation time, TTC concentration and pH. In any case pH must be kept under 12, higher pH values can induce TTC chemical instead of enzymatic reduction (Lin et al., 2001). The elimination of bleach sterilization step and the reduction, or even elimination, of detergent seems to ameliorate the results of the method with chlorophyllic spores, such as *O. regalis*,, but further work is needed. Thus, in the case of green spores, bacterial and fungal contamination should be evaluated independently. TTC reduction is sensitive to light and oxygen concentration (Towill and Mazur, 1975), therefore TTC incubation must be performed in the absence of light and without agitation.

Treatment with 95% ethanol (Towill and Mazur, 1975; Chen et al., 1982; De Block and De Brouwer, 2002) or distilled water (Lin et al., 2001) as assayed by others in plant tissues, is not enough to extract TPF precipitates from the mitochondria of the spore. The use of DMSO (Backor and Fahselt, 2005) resulted in the extraction of higher levels of contaminant pigments. The procedure using the mixture 0.1 N KOH:EtOH 96%, 1:1, *v*/*v* (Steponkus and Lanphear, 1967; French and Parkin, 1993), yielded better results but still some vigorous procedures had to be introduced in order to optimize TPF recovery. The measurement of the red coloured water-insoluble TPF salt is impeded by the interference of pigments, such as chlorophyll, co-extracted into the ethanol (Steponkus and Lanphear, 1967). TPF salts can be partitioned

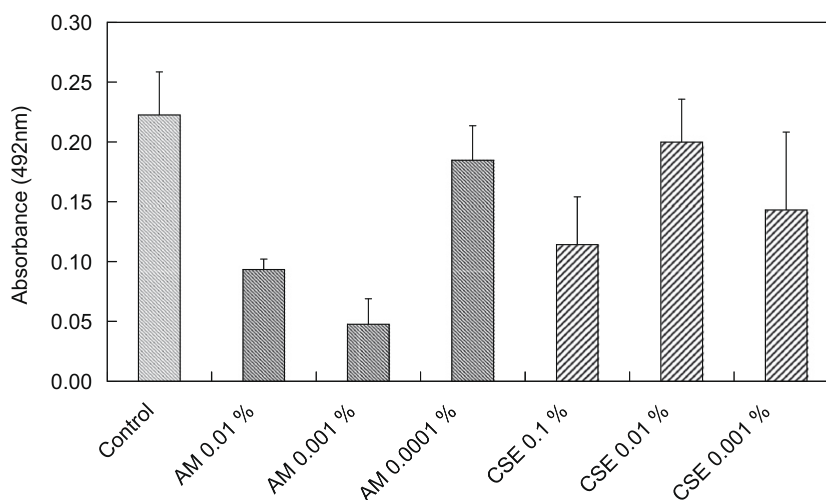

Fig. 5. Effect of 24 h incubation with different substances on *D. guanchica* spore mitochondrial activity. Cells were incubated during 24 h in autocleaved Dyer–Tween medium containing different concentration of pollutants (AM aromatic mixture, CSE contaminated soil extract), at 20 °C and 8 h/16 h photoperiod. Then mitochondrial activity was evaluated in 2.5×10^4 spores, incubated with TTC 0.6%, pH 8 during 1 h in darkness.

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away by prompt addition of hexane (French and Parkin, 1993), nevertheless we observed a slight contamination of the hexane phase with unwanted plant pigments absorbing at 492 nm. This effect increases with the number of cells used but the absorbance due to contaminants is very low and the use of a simple blank sample with the same number of spores and TTC 0% in every assay can minimize this factor.

We also found that the use of *n*-hexane extraction increases TPF photosensitivity turning it from red to yellow. So we recommend protecting the samples from light, at room temperature, since the process is accelerated at low temperatures, and measuring absorbance immediately after extraction. Many formazans can be obtained in red and yellow forms on exposure to visible light (Hausser et al., 1949). The change to yellow on irradiation occurs very slowly in polar solvents as ethanol, compared to non-polar solvents such as benzene, where the change is complete after several minutes. It is probable that the yellow product is a formazan with a broken hydrogen bond (Hausser et al., 1949; Nineham, 1955).

Mitochondrial activity is a very sensitive biomarker directly related to viability. Environmental stressors disturbing mitochondrial activity will affect the spore/gametophyte energy budget potentially jeopardising organism survival. A reduction of competent cells due to damage or toxicity will diminish the total amount of formazan salts reduced at mitochondrial chains. This constitutes the basis for bioassays. The preliminary study on polycyclic hydrocarbons toxicity presented in our work demonstrates that the evaluation of mitochondrial activity in fern spores or gametophytes can be used as a bioassay for environmental toxicology. Nevertheless, plant mitochondrial activity is a biomarker known to undergo hormetic effects (Calabrese, 2008). Hormesis is a dose–response phenomenon that is characterized by low-dose stimulation and high-dose inhibition (Calabrese and Blain, 2009). We have recently obtained data from spore bioassays confirming the occurrence of hormesis with different substances and environmental samples. These results will be published elsewhere.

The fern spore bioassay presents several practical advantages: easy recollection and manipulation of test organism, low cost, only basic lab equipment needed, relatively short execution time and easiness of adaptation to high throughput technologies (i.e. automated analyser, microplate reader). Despite the simplicity of the method, when we compare the fern spore bioassay based on mitochondrial activity with other bioassays we find further advantages:

1. Biological relevance: ferns are higher plants. The conclusions obtained are highly relevant for other eukaryotic organisms, especially in the case of higher plants such as crops or wild plants. Comparable bioassays based on prokaryote organisms render results that are not easily extrapolated to eukaryote organisms.
2. Ecological relevance: we can use spores of ferns belonging to different habitats. For example, the use of spores from a river-bank fern, such as *P. setiferum* in a bioassay, provides information about possible effects of a pollutant on the river bank habitat.
3. *Versatility:* With the same organism and methodology, different types of bioassays would be possible: acute toxicity (24–72 h) or chronic toxicity (weeks).
4. Fern spores can be easily transported and preserved at 4 °C for months or even years and do not need continuous growth or feeding such as other organisms.
5. Very low volume of test sample needed. If the samples can be analysed in a microplate reader, less than 1 mL of test sample is needed.
6. In contrast with other cell bioassays that use clone populations (algae, bacteria, cell lines or primary cultures), fern spores are meiotic products and thus every spore is genetically different from another. This endows the method with higher biological relevance since plant wild populations present a natural genetic diversity.

Finally, this model sums up the advantages of methods based on single cells with those of pluricellular organisms. In a short time, spores give rise to gametophytes that may be used as a pluricellular plant model with no further modifications on the method. These features make this method suitable for commercialization in the shape of a kit.

5. Conclusion

Here we show that the assessment of fern spores mitochondrial activity could constitute a useful bioassay for environmental toxicology. We present in this work a simple, rapid and inexpensive means of assessing viability of fern spores and gametophytes when dealing with large number of samples based on the reduction of TTC to TPF coloured salts. *D. guanchica* is a good model species for the study of mitochondrial activity of fern spores for its high production of spores, the extended period of recollection and the easiness of manipulation of the spores. Procedures selected as optimum in the model species *D. guanchica* are as follows: bleach sterilization, incubation without shaking at 20 °C in the dark for 1–4 h with 0.05–1.5% TTC in Dyer medium supplemented with 0.001–0.005% Tween 20 at pH 8.

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