Anal Bioanal Chem (2010) 398:1883–1889
DOI 10.1007/s00216-010-4130-9

ORIGINAL PAPER

Fiber-optic pH detection in small volumes of biosamples

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Received: 2 May 2010 / Revised: 13 August 2010 / Accepted: 15 August 2010 / Published online: 12 September 2010
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Abstract Determining the pH values of microscopic plant samples may help to explain complex processes in plants, so it is an area of interest to botanists. Fiber-optic probes with small dimensions can be used for this purpose. This paper deals with the fiber-optic detection of the pH values of droplets of plant xylem exudate based on ratiometric fluorescence intensity measurements with an internal reference. For this purpose, novel V-taper sensing probes with a minimum diameter of around 8 μm were prepared that enable the delivery of fluorescence signal from the detection site on the taper tip to the detector. The taper tips were coated with pH-sensitive transducer (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt; HPTS) and a reference [dichlorotris-(1,10-phenanthroline) ruthenium (II) hydrate (Ru-phen dichloride)] immobilized in a xerogel layer of propyltriethoxysilane and (3-glycidoxy)propyl trimethoxysilane. The prepared probes were sensitive to pH values mainly in the range from 6.0 to 9.0. In the pH range 6–9, the results were limited by measurement errors of about 0.2 pH units, and in the pH range 5–6 by measurement errors of about 0.5 pH units. Using the developed V-taper sensing probes, the pH values of in vivo and in vitro samples of small volumes (~6 μl) of exudate were measured. The results were validated by comparison with conventional electrochemical pH measurements.

Keywords Fluorescence · pH · HPTS · Exudate · V-taper sensing probe

Published in the special issue *Optical Biochemical and Chemical Sensors (Europtrode X)* with Guest Editor Jiri Homola.

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Introduction

The determination of the pH values of bulk samples or microscopic biosamples has been of interest to botanists for many years. A knowledge of the pH values or pH gradients in biosamples can help to explain complex processes in plants [1, 2]. Local pH differences in plant tissues can modulate the activities of enzymes involved in the metabolism of bioactive compounds controlling plant growth and development. As an example, two different isozymes catalyzing the degradation of the plant hormone cytokinin (cytokinin oxidase/dehydrogenase) differ significantly in their pH optima (6.5 and 8.5, respectively), enabling the pH-dependent regulation of cytokinin levels, which—in cooperation with another plant hormone, auxin—control cell division and differentiation [3]. Further, it has been suggested that the concentration of another plant hormone, abscisic acid, which controls the opening of stomata and transpiration, is affected by the partitioning of the hormone between the symplast and apoplast in response to the alkalization of xylem samples in plants exposed to water deficit [2], [4].

The pH values of biosamples can be determined electrochemically by means of conventional pH meters with ion-selective electrodes [5] which are scalable to micrometer size [6, 7] or optically. The pH values of bulk biosamples can be determined optically, for example by spectroscopy, after entrapping suitable optochemical transducer(s) in solution

[8, 9]. The pH values of microscopic biosamples are widely determined with microscopes using fluorescent labels (markers, transducers), which allow the pH distribution and temporal changes in pH to be monitored. A lower contrast and the autofluorescence of biosamples can be considered drawbacks of this approach, and they can be diminished by using a confocal microscope [2], [10, 11]. Nanoparticles containing optochemical transducers can also be implemented into biological microsamples and used for chemical sensing [12, 13]. These probes, encapsulated in biologically localized embedding (PEBBLEs), are submicron-sized sensors which enable the monitoring of analytes in viable cells using a fluorescence microscope. Recently, optical nanosensors have been investigated for chemical sensing in biological microsamples such as animal or plant cells [14–16]. The advantage of these nanosensors is their nanosize, which enables the intracellular sensing of physiological and biological processes. A special technique, near-field scanning microscopy, has been used to achieve fine spatial distribution.

The pH values of microscopic biosamples can also be determined by means of optical sensors based on fiber-optic probes [17, 18], particularly when real-time, noninvasive and/or remote monitoring is required. Since the first demonstration of pH fiber-optic detection for physiological use in the 1980s [19, 20], a number of principles (e.g., absorption reflection, fluorescence, time-domain fluorescence, evanescent wave, etc., as described in a comprehensive overview [21]) have been developed. These sensors have been widely used in the laboratory or field, mainly in relation to biotechnology [22] and medicine [23].

Fiber-optic probes usually employ light from the detection site. The detection site is placed on the taper tip, which contains an optochemical pH transducer. Although absorbance-based pH sensors can be employed for measurements in biological microsamples [24], fluorescence transducers are now preferred [25]. Transducers such as 2′,7′-bis(2-carbonylethyl)-5 (6)-carboxyfluorescein (BCECF) [21], [26], 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) [27, 28] or 6,8-dihydroxy-1,3-pyrenedisulfonic acid disodium salt (DHPDS) [29, 30] have been used for detection in the biologically important pH range from 5 to 7. Direct fluorescence intensity measurements can suffer due to fluctuations in the temperature or the excitation source or variations in the experimental arrangement (e.g., variations in sample size). Therefore, an approach based on fluorescence lifetime measurements [31] or based on referencing via ratiometric measurements has been developed.

Present fiber-optic pH-sensors (e.g., [24]) are usually based on multimode silica fibers of diameter 100–300 μm terminated with a larger sensing head. These dimensions allow users to analyze samples with volumes of tenths of a milliliter. However, tapering fiber-optic probes to a diameter of several microns allows the real-time monitoring of much smaller samples, such as xylem droplets with volumes of several microliters, directly at their site of exudation. Such an approach minimizes changes in pH values during microsample collection [2] and aids the investigation of plant life processes.

This paper deals with the preparation of tapered fiber-optic sensing probes and their employment for the fluorescence detection of pH in droplets of plant exudate. The sensing probes are used not only to deliver the excitation signal to a transducer on the fiber tip but also to collect the emitted fluorescence and deliver it to the detector. Ratiometric measurements with an internal reference of a Ru-phen dichloride are used.

Experimental

Since droplets of exudate are produced by living plants, a fluorescent transducer HPTS with a known optical response to pH in the range 5–7 (corresponding to pH values in living plant materials) was employed [32]. Dichlorotris-(1,10-phenanthroline) ruthenium (II) hydrate (Ru-phen dichloride) was chosen for use as an internal reference; it produces fluorescence at longer wavelengths than HPTS. Both of the transducers can be effectively excited at around 475 nm.

Novel fiber taper probes were prepared by tapering twin polymer-clad silica (PCS) fibers of diameter 125 μm or graded-index (GI) fibers 50/125 μm [33], and V-taper probes were produced by careful cutting the tapers around their waists [34]. V-taper probes with waist diameters ranging from 8 to 40 μm were prepared in this way and used in experiments. The transducers were pre-complexed and immobilized onto the tips of the V-taper probes using propyltriethoxysilane (PTES) layers modified with (3-glycidoxy) propyltrimethoxysilane (GLYMO). Five milligrams of a complex of HPTS (Aldrich), prepared by the technique described in [27], was dissolved under regular stirring in a mixture of 5.5 ml PTES (Aldrich, >98%) and 5 ml of ethanol (Sigma-Aldrich, ACS reagent) to form a transparent solution. A mixture of 1 ml of ethanol, 1.35 ml of deionized water and 100 μl of HCl (Alfa, semiconductor grade) was added dropwise under regular stirring, and the resulting solution was thermally treated in an oil bath at 85 °C for 30 min. The solution prepared was cooled down to laboratory temperature and 5.4 ml of GLYMO (Fluka, purum, >97%) was added dropwise into the solution. Finally, 5 mg of Ru-phen dichloride were added to the solution, which was ultrasonicated for 10 min and aged at laboratory temperature for 24 h before deposition. The resulting sol was applied onto the taper tips by a dip-coating technique. The layers prepared in this way were thermally cured at 80 °C for 12 h, and the V-taper sensing probes produced were calibrated and used for measurements of plant microsamples.

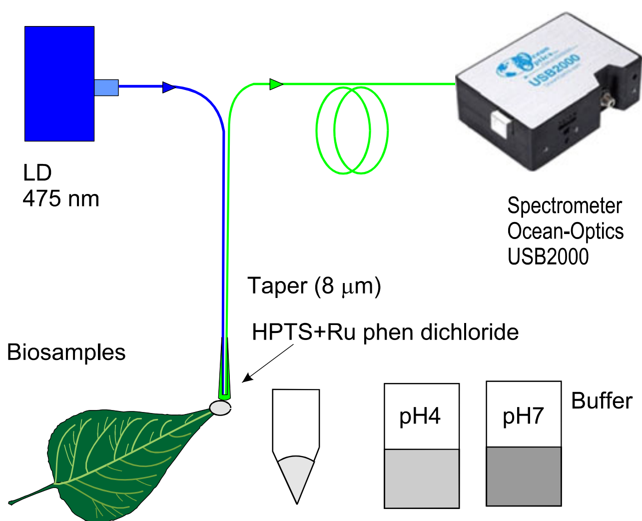

Fig. 1 Experimental set-up for pH measurements comprising a laser-diode source, a pocket fiber-optic spectrometer and a fiber-optic probe tapered to a diameter of 8 μm and coated with HPTS and Ru-phen dichloride transducers

The V-taper sensing probes were calibrated using Britton–Robinson colorless buffer solutions of different pH values but the same ionic strength (0.15 mol/l). The pH values of the buffer solutions were determined with a conventional pH meter (Jenway 3305), calibrated using standard pH buffer solutions of pH 4.01 and pH 7.01 (Carl Roth GmbH). The optical responses of the V-taper sensing probes were characterized using the experimental setup depicted in Fig. 1.

The calibration curve was determined from fluorescence spectra by dividing the maximum fluorescence intensity of HPTS by the maximum fluorescence intensity of Ru-phen dichloride (after subtracting the background optical output power obtained for pH 4). Each assay of optical output power for calibration curve measurements was accompanied by two check assays. The integration time of these measurements was optimized at 0.5–1 s. The calibration curve was plotted on the basis of average values, and error bars were added, including the confidence limits.

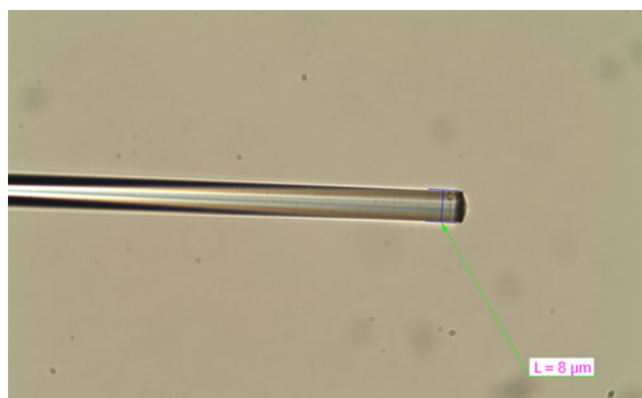

Fig. 2 V-taper sensing probe made from twin PCS fibers of 125 μm diameter tapered to a diameter of 8 μm

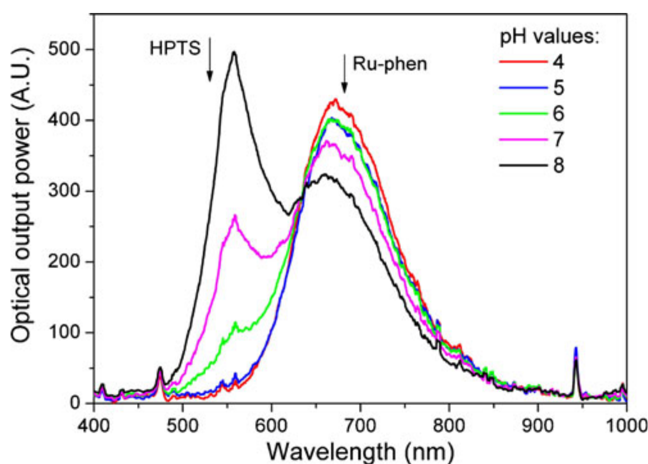

Fig. 3 Fluorescence responses of the V-probe of diameter 8 μm with immobilized HPTS and Ru-phen dichloride to the pH values of various buffers

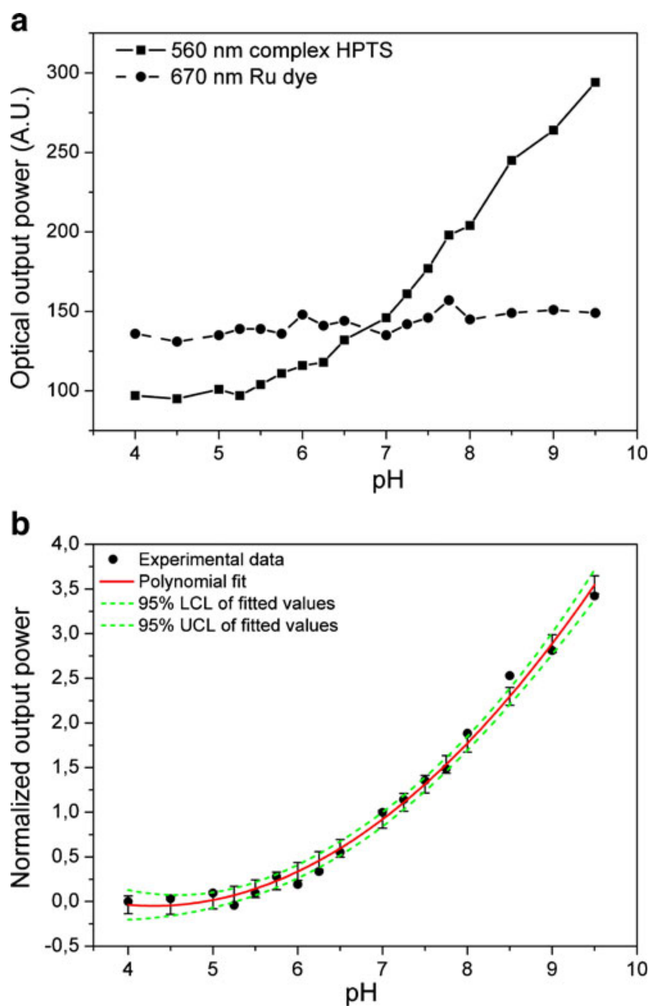

Fig. 4a–b Calibration curves of the V-taper sensing probe in response to pH changes: **a** optical responses of the HPTS transducer and Ru-phen dichloride to pH; **b** calibration curve (including upper and lower confidence limits, *UCL* and *LCL*, respectively)

Data gathered for the calibration curve were fitted with a second-order polynomial given by the general equation

$$normalised_output_signal = A + B \cdot pH + C \cdot (pH)^2. \quad (1)$$

The sensitivity of the optical sensor was expressed by the general equation [35]

$$sensitivity = \frac{d(sensor_response)}{d(measured_quantity)} = \frac{d(normalised_output_power)}{d(pH)}. \quad (2)$$

The V-taper probes were used to determine the pH of exudate in vivo and in vitro. Individual droplets of exudate naturally excreted by oat plants cultivated in a ClimaBox were analyzed (in vivo) at first, and then the measured droplets were collected and the integral value of the bulk sample was analyzed again (in vitro). The sensor response was regularly checked in standard buffers of pH 4 and pH 7 after every five samples. The droplets were ~0.5–1 mm (a volume of several microliters) in size, and this size depended on the exposure time of the plants from the ClimaBox. It was not possible to determine the pH values of individual droplets several times because of their lifetimes; collected bulk samples were determined three times, and mean values are presented.

Finally, the pH of a selected in vitro sample with a volume that was sufficient for conventional pH measurements was validated using a pH meter. Each of the pH values determined electrochemically and optically was measured three times, and mean values are presented. In order to validate the pH values obtained electrochemically, several pH meters were used with deviations of±0.08 pH units.

Results and discussion

Novel V-taper sensing probes that are capable of effectively guiding weak fluorescent signals from the detection site at the taper tip to a detector were designed and prepared from PCS fiber (see Fig. 2). Similar probes have also been prepared by tapering standard graded-index fibers. The mechanical durability of the prepared probes allowed us to penetrate into real sample drops for tens of measurement cycles over a period of several weeks.

Fluorescence spectra obtained from the V-taper sensing probe when excited by the laser diode (LD) can be seen in Fig. 3. The fluorescence spectra include a broad emission peak from HPTS with a maximum at around 560–570 nm and a peak from the reference Ru-phen dichloride with a maximum around 670 nm. The peak from the LD used for excitation and its first harmonic can be observed at around 475 nm and 950 nm, respectively.

The fluorescence intensity of HPTS is strongly dependent on the pH of the calibrating buffer down to a limiting value of pH 5.0, while the fluorescence intensity of Ru-phen dichloride remains similar across the range of tested pH values (Fig. 3, 4a). Values of the fluorescence intensity depend on the waveguiding conditions of the V-taper sensing probe and the surrounding analyte, as can be seen by comparing Fig. 3 and Fig. 4a. These variations can be attributed to changes in the optical conditions of individual measurements, such as changes in the refractive index of the analyte, the probe used, and the bending of the probe during measurement, etc. In order to diminish this effect, the fluorescence peak of Ru-phen dichloride was employed as an internal reference (Fig. 4a).

The calibration curve can be seen in Fig. 4b. The dependence of the optical output power on the pH was fitted by the second-order polynomial shown below. The parameters of the fit and their corresponding errors are summarized in Table 1.

$$normalised_output_signal = 2.40722 - 1.14235 \cdot pH + 0.13288 \cdot (pH)^2. \quad (3)$$

The nonlinear response of the sensor implies that the sensitivity is not constant across the full measured pH range. Considering the experimental calibration curve of the sensor plotted in Fig. 4b, and the corresponding polynomial fit represented by Eq. 3, the sensitivity of the sensor can be expressed as the first derivative of Eq. 3:

$$sensitivity = -1.14235 + 2 \cdot 0.13288 \cdot (pH). \quad (4)$$

For example, the calculated sensitivities for pH values of 5.5 and 7.5 are 0.32 pH^{-1} and 0.85 pH^{-1}, respectively. The error in the sensor response evaluated from the calibration curve in Fig. 4b for a confidence level of 95% is lower than 0.16 units. The experimental error in the pH measurements can be evaluated from these data as the error in the sensor response

Table 1 Parameters of the fitted calibration curve

A		*B*		*C*		
Value	Standard error	Value	Standard error	Value	Standard error	Adj. *R*-square
2.40722	0.48514	−1.14235	0.1481	0.13288	0.01089	0.99001

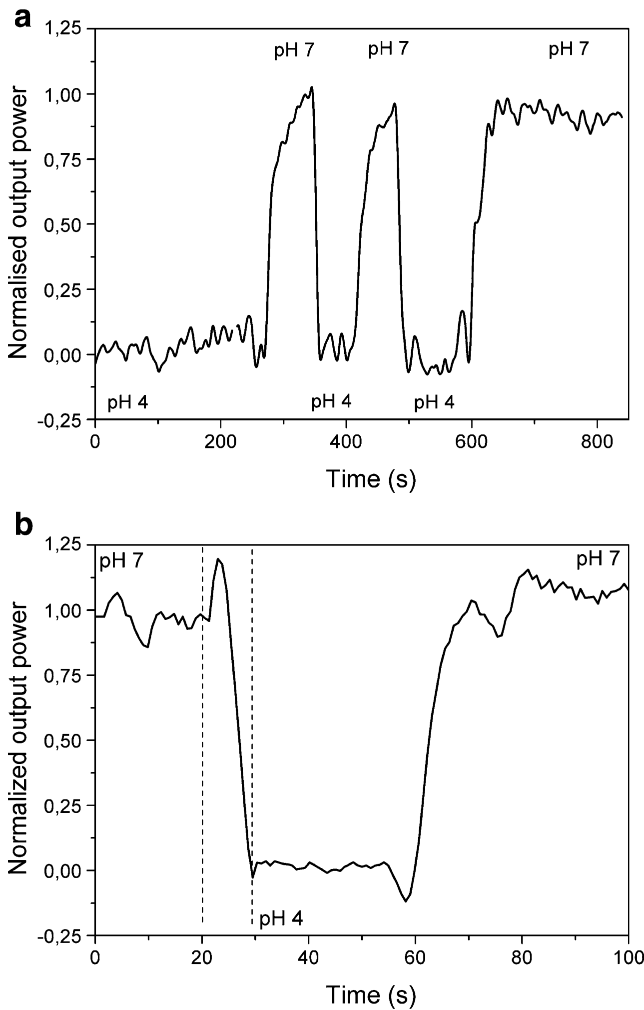

Fig. 5 **a** Medium-term stability and repeatability; **b** Dynamic response between two values of pH

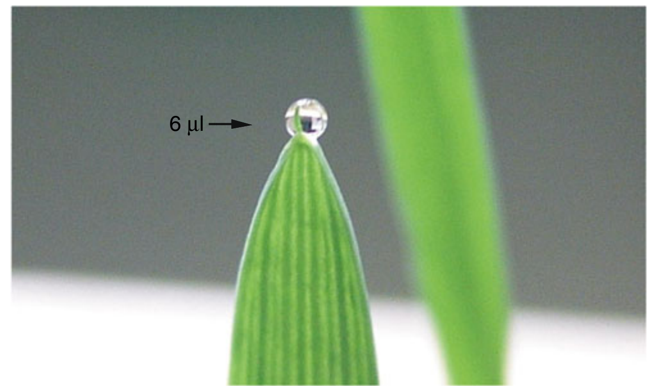

Fig. 6 Guttation of xylem sap from a primary leaf of oat

divided by the sensitivity: 0.16/0.32~0.5 pH units in the pH range 5–6 and 0.16/0.85~0.2 pH units in the pH range 6–9.

The dynamic response and reproducibility of the V-taper sensing probe were measured through the cyclic immersion of the probe into two Britton–Robinson buffers of pH 4 and 7. Despite the uncontrolled temperature during the experiments, the measured signal was characterized by good reproducibility and time stability without significant drift, as can be seen from Fig. 5a. Concerning the dynamic properties of the sensor probe, the response was shorter than 10 s, including the time needed to change buffers. The V-taper sensing probe was stable for tens of measurement cycles taken over a period of several weeks. Crucial parameters influencing its stability were the content of GLYMO in the starting sol, and the time and duration of the curing of the sensing layer. This V-taper sensing probe, transducer included, was primarily designed for the detection of pH between 5 and 7, a range that is relevant to the conditions that exist in samples investigated by botanists, such as in living plants. However, interaction with strong alkali leads to the decomposition of the sensing layer, entrapping the optical transducer.

Using the calibration curve (Fig. 4b), the pH values of small droplets of exudate (in vivo) and exudate collected from the droplets (in vitro) were determined (Table 2, Fig. 6).

Nearly all of the pH values of the measured samples fall within the range 4.9–6.0 pH units. The pH values of individual droplets measured in vivo were, within experimental error, in agreement with the pH values of the corresponding collected and pooled samples measured in vitro. Despite the low sensitivity in the determined pH range, the method allowed us to distinguish small differences in the pH values of xylem exudates collected from different leaf parts. Compared to the results obtained using other plant species and other methods of determining pH [2], no profound vertical pH gradient was found in primary oat leaves. Obviously, the in vitro measurements allow better sample accessibility, reducing the experimental errors introduced during measurement. The pooling of samples could affect the pH values of the solutions due to

Table 2 pH values of individual droplets and pooled samples of xylem exudates from different parts of the first leaves of oat (*Avena sativa* L., cv. Abel)

	Guttation solution excreted at the top tip of the leaf	Xylem exudate excreted after cutting off a leaf tip	Xylem exudate excreted after cutting off the leaf at its morphological base
Mean pH of droplets	5.0	5.6	5.5
Standard deviation of mean droplet pH	0.3 (9 droplets)	0.3 (5 droplets)	0.1 (6 droplets)
pH of pooled solution	5.4	5.4	6.0

their storage (even for a short time period) and the potential affinities of some sample constituents for the surface of the container.

Finally, two samples of exudate from a third independent set of collected reference samples were used to validate the results. First, the pH of each sample was determined by the V-taper sensing probe, and values of 5.6 and 5.5 were obtained. Then the pH values of the corresponding samples were determined by a conventional pH meter, and values of 5.3 and 5.0, respectively, were obtained. Taking into account the experimental error associated with a conventional pH meter (±0.08) and the experimental error associated with the V-taper sensing probe in this pH range (~0.5), the optical approach developed here can be considered to be validated.

When applying the novel fiber-optic probe, the same pH analytical procedure established in experimental botany [2], [5] was employed, which takes into account interference from the ionic strengths of analytes and other influences. The proposed ratiometric concept restrains the influence of temperature variations to a minimum, as the value of the output optical power originates from a comparison of the fluorescence at two wavelengths obtained at the same temperature. The near-constant character of the fluorescence of Ru-phen at 670 nm during measurements (Fig. 4a) indicates the effective inhibition of interference from oxygen during pH measurement.

Conclusions

Novel V-taper probes with a minimum diameter of around 8 μm that were coated with a pH-sensitive transducer (HPTS) and an internal reference (Ru-phen dichloride) were prepared. The probes were sensitive to pH mainly in the range from 6.0 to 9.0. In the pH range 6–9, the results were limited by measurement errors of about 0.2 pH units, and in the pH range 5–6 by measurement errors of about 0.5 pH units. Such measurement errors are mainly determined by the optochemical properties of the HPTS transducer employed. The developed probes were used for pH detection in in vivo and in vitro microsamples of exudate with volumes as low as 6 μl. Mean pH values measured in vivo on individual droplets produced on the tips of oat leaves were between 5.0 and 5.6, depending on the region of the leaf, and these values correlated well with the combined value for the collected samples. The results of the optical measurements were validated by comparison with values determined by an electrochemical pH meter on in vitro samples. The values obtained using the different techniques agreed well within experimental error. Further prospects for the presented fiber-optic method include the measurement of pH using probes that are directly inserted into intercellular (apoplastic) and intracellular (symplastic) spaces.

Acknowledgement This research was supported by the Ministry of Education, Youth and Sport of the Czech Republic (contract No. LC06034), by Grant Agency AS CR (grant No. IAA600380805), and by the National Science Foundation (contract no. 102/08/P639).

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