3D STUDIES OF *OROBANCHE CUMANA* SEEDS BY DIGITAL HOLOGRAPHIC MICROSCOPY

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Abstract — **Combining microscopy and digital holography (DH) offers the unique advantage of simultaneously capturing complete 3D information about the specimen, and under coherent illumination it has been investigated for continuous PC acquisition and identification of biological specimens.**

Sunflower (*Helianthus annuus* L.) broomrape *Orobanche cumana* Wallr.(*O. cumana*) is a parasitic angiosperm, totally devoid of chlorophyll, that infects the roots of sunflower plants, causing significant damages to this strategic oilseed crop in Black Sea countries as Turkey, Romania, Ukraine, Bulgaria, including Republic of Moldova (RM). The long-term impact of the broomrapes is even more serious: their seeds may easily spread to other fields, and can persist in soil up to 20 years, leading to an accelerated increase in the infested areas.

The aim of this research is to develop three-dimensional microscopy (3D) system to enhance the resolution and contrast in imaging of *O. cumana* seeds, occurring in different geographical regions of RM, Romania, Spain and Ukraine. Successful application of the modern technology allowed studying the broomrape seed in various layers, like surface and under surface of seed that can be the reliable means to distinguish related taxa and their geographical localization.

Index Terms **— Digital holographic microscopy, optical and digital image processing, *Orobanche cumana* seeds, biological specimen.**

I. INTRODUCTION

In the past decade 3D optical imaging has emerged as a leading tool for scientific discovery in many different fields, including biology. Optical image processing techniques are very useful thanks to their advantages such as high spatial resolution, parallel processing, non-contact parallel communication, and direct visual presentation to human eyes.

Optical imaging system using DH [1] is an attractive technique for the acquisition of 3D information by reconstructing both the amplitude and phase of the wave front that passes through or is reflected / scattered by the object. In order to establish DH in microscopy, the combination with common microscopy techniques is of particular advantage. In this case flexible and compact Digital Holographic Microscopy (DHM) modules for the integration into modern microscopy systems are required. Furthermore, for an automated evaluation of the measurement data, it is necessary to implement robust algorithms for the numerical reconstruction of digital holograms. Combining microscopy and DH offers the unique advantage of simultaneously capturing complete 3D information about the specimen, and under coherent illumination it has been investigated for continuous PC acquisition and identification of biological specimens. In contrast to conventional microscopy, no depth scanning focusing at various depths of the 3D specimen are necessary in range of some interference fringes.

The lateral resolving power of an optical system is diffraction-limited according to Abbe Criterion. Considering the fact that complex biological specimens consist of different materials or structures in various layers, there are some practical obstacles to prevent achieving the theoretical resolution, e.g. the diffraction patterns coming from the upper layers.

A basic digital holographic microscopy setup consists of a laser illumination source, an interferometer, a digitizing camera (CCD or CMOS), and a computer with necessary programs [1, 2]. One of the major issues in imaging the internal structures of biological specimens is the absorption and scattering that distort the information coming from the object. Dark-field technique has been shown to be promising in improving the image contrast for internal layers in combination with optical sectioning techniques [3] and digital holographic refocusing [4].

Digital holography offers a number of significant advantages, such as the ability to acquire holograms rapidly, availability of complete amplitude and phase information of the optical field, versatility of the interferometric and image processing techniques [5].

Imaging internal layers of biological specimens is a demanding field of research [6]. DHM is a combination of holography and microscopy and has ability to extract essential 3D information from a single recording. With the help of numerical reconstruction and digital focusing DHM has become a new tool for biological specimens imaging.

Broomrape is a parasitic angiosperm that has been causing a great deal of damage to sunflower production in many countries, including Republic of Moldova. Control of this parasite remains extremely difficult, as thousands of tiny seeds produced by a single broomrape plant can be easily dispersed by water, wind, animals, humans, machinery or

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attached to sunflower seeds. Broomrape seeds survive in soil as miniature dormant plants much longer than the active, significantly larger, mature parasites — they often persist in soil for decades, and thus maintain the parasite ability to mature whenever a suitable host plant occasionally grows nearby [7, 8]. This ability, which is crucial for the continual existence of obligate parasites, is facilitated by structural features that accommodate dormancy and dormancy control [9]. Seeds of broomrape are extremely small, being less than 1 mm in size, with a wide variety of shapes (ellipsoid, oblongoid, ovoid, globose, trigonous or tetragonous) and a terminal funicular attachment [10]. The seed coat is reticulated with polygonal cells, which range from more or less isodiametric to tangentially elongated, being sometimes irregular.

Species distinction in *O. cumana* L. is notoriously difficult because of the lack of clear cut morphological differences. References [11], among others, have stressed the great importance that the sculpturing of the seed can have for broomrape taxonomy. The micromorphology of the *Orobanche* genus seed surface is highly diverse and has been described for various species, particularly of the genera that are economic importance. Seed coat sculpturing can be helpful in the analysis of soil seed banks, but due to the variations in seed shape, size and ornamentation even within a single capsule, unequivocal morphological diagnosis should rely on a set of seeds rather than on a single one [12, 13, 18].

The aim of this research is to develop three-dimensional microscopy system to enhance the resolution and contrast in imaging of broomrape seeds, occurring in different geographical regions of RM, Romania, Spain and Ukraine.

II. Methods and Materials

Seed morphology was studied in 13 populations of the *O. cumana* from different geographical regions of the Republic of Moldova (Donduseni, Soroca, Balti, Chisinau, Singera, Rezeni, Cimislia, Stefan Voda, Ciadir-Lunga, Taraclia), Romania (Fundulea), Ukraine (Izmail) and Spain (Sevilia).

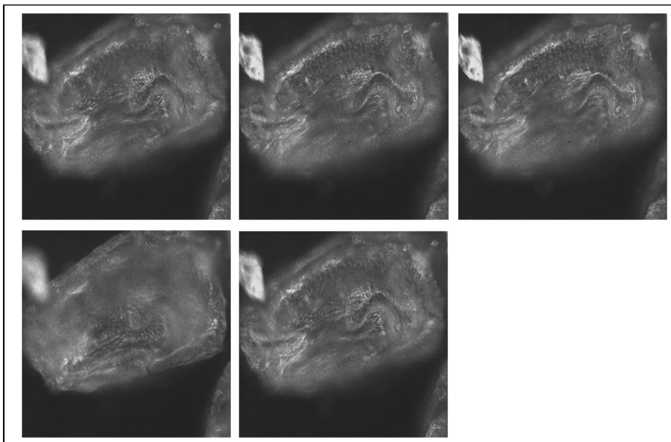

Fig. 1. Dark-field holographic micrographs of *O. Cumana* seeds (Singera) on reflection mode of illumination (λ=740nm).

Morphological observations of seeds were made using a dark-field holographic microscope. For the dark-field imaging, a laser wavelength of 740 nm was utilized. Pictures were taken with reflection and transmission settings. To focus on different surface regions of the seeds, manual adjustments were done varying the distance between front objective lens and biological specimen. On Fig. 1 we present digitally reconstructed images of the different surface regions placed at focus on reflection mode of illumination, which allows more detailed studying of specific seed's region of interest. In addition one of the optical pathways was covered to change transmission to reflection and vice versa. Dark-field holographic micrographs of *O. Cumana* seeds (Singera) on transmission mode of illumination (λ=740nm) are shown on Fig.2. From comparison data of reflection and transmission mode of illumination one can see that reflection gives us information about surface morphology of seeds, but transmission illumination penetrates under surface. Varying the plane under focus we see different layers of inner structure of sample.

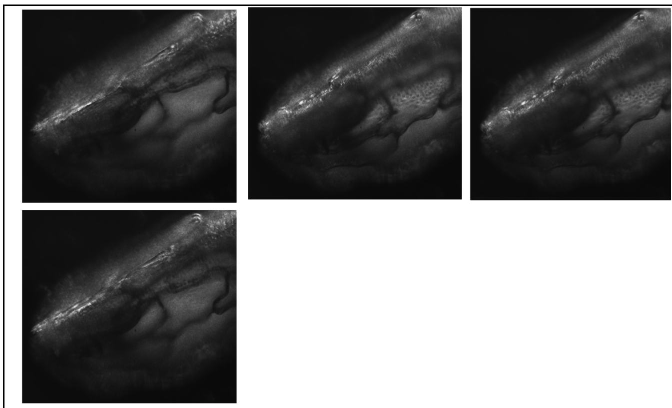

Fig. 2. Dark-field holographic micrographs of *O. Cumana* seeds (Singera) on transmission mode of illumination (λ=740nm).

During the experiment pictures of ten seeds per category, randomly chosen, were taken using transmission and reflection mode.

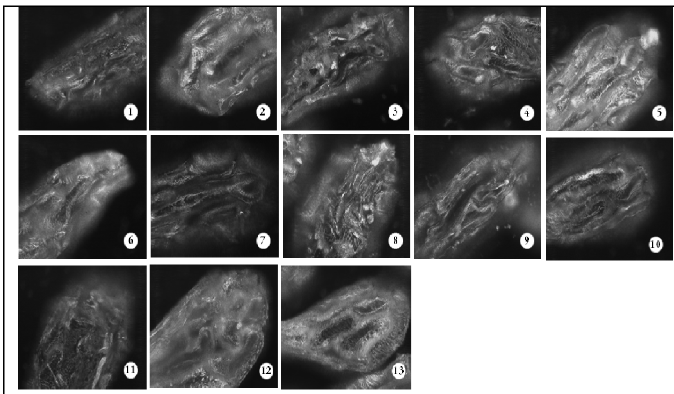

Fig. 3. Dark-field holographic micrographs of *O. cumana* seeds on reflection mode of illumination (λ=740nm). 1 – Singera, 2 – Stefan Voda, 3 – Chisinau, 4 – Donduseni, 5 – Rezeni, 6 – Balti, 7 – Ismail, 8 – Ceadir-Lunga, 9 – Cimislia, 10 – Taraclia, 11 – Fundulea, 12 – Bacioi, 13 – Sevilia

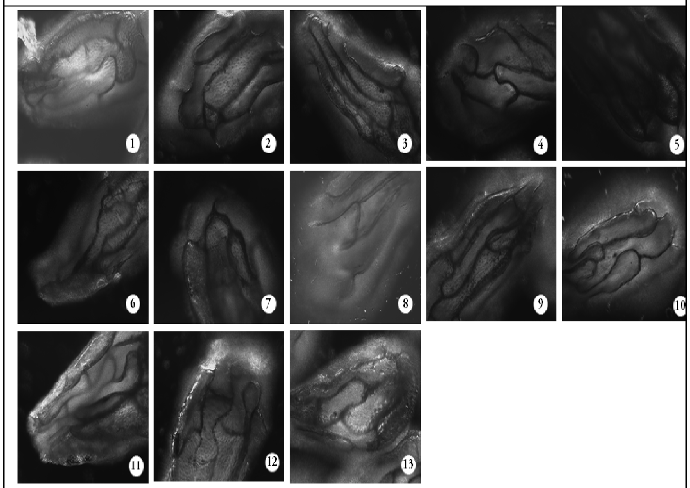

Fig. 4. Dark-field holographic micrographs of *O. Cumana* seeds on transmission mode of illumination (λ=740nm). 1 – Singera, 2 – Stefan Voda, 3 – Chisinau, 4 – Donduseni, 5 – Rezeni, 6 – Balti, 7 – Ismail, 8 – Ceadir-Lunga, 9 – Cimislia, 10 – Taraclia, 11 – Fundulea, 12 – Bacioi, 13 – Sevilia

III. Results and Discussions

Analysis of seed photomicrographs of the 13 geographical populations of *O. cumana* obtained using digital holographic microscopy allowed to investigate the architectural surface of the seeds. The high quality of the images revealed the broomrape seed surface sculpturing.

In this work we have shown that imaging through opposed-view channels, including reflection (Fig. 3) and transmission (Fig. 4) modes, reveals more structures of an internal layer of the specimen.

The broomrape populations exhibit a variety of different shapes and surface characteristic. These morphological characteristics, and in particular seed sculpturing, are key features in determining of their geographical localization.

The biological samples, as a rule, possess very complex structure and layers-like morphology all these hinder achievement of the theoretical optical resolution. The most important obstacle here is the diffraction on biological spacemen structure. In this work it is developed a coherent light microscope was elaborated to get over the obstacle.

The multi-purpose set-up has been composed from four off-axis digital microscopes in one (Fig.5). It gives possibility to acquire digital holograms in two modes of illumination; to wit, dark-field and bright-field in transmission /reflection configurations. Two single mode diode lasers with wavelengths λ=660nm and 405nm could be applied for sample illuminations. Optical properties of biological sample determine the wavelength selection and mode of illumination. The dark-field image acquisition mode by illumination with λ=660nm in transmission and reflection mode is a good solution for biological sample with high scattering properties. The bright-field image acquisition at λ=405nm gives a higher resolution and contrast due to such properties of biological sample as high absorption.

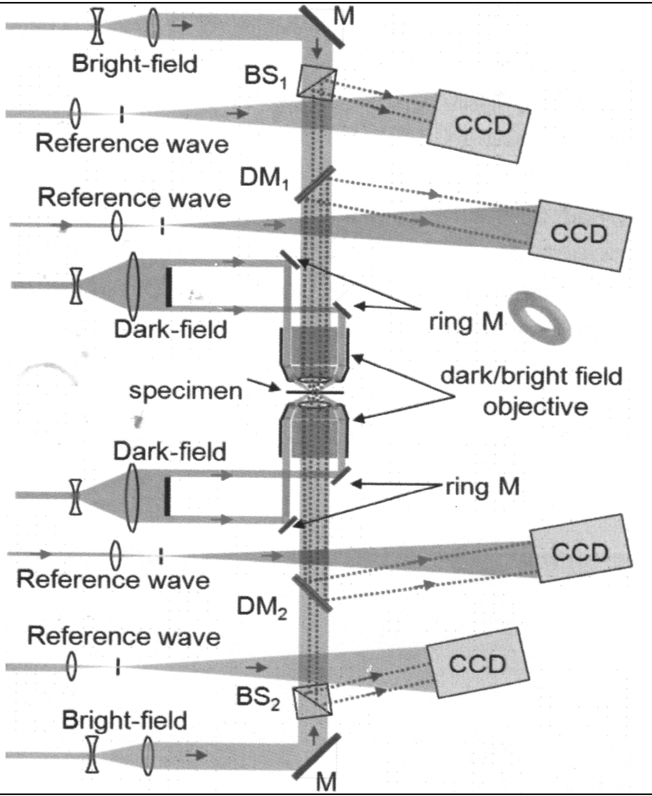

Fig.5. Schematic diagram of the imaging part of the opposed-view set-up, showing both dark-field and bright-field configurations.

A pair of Nikon dark/bright-field objectives with magnification of 20x and NA=0.45 has been implemented in the set-up. Hollow light cone of illumination through parabolic mirror of objective has been used for dark-field mode of illumination. Illumination through objective lens was applied for bright-field illumination.

CCD cameras simultaneously acquire images in transmission and reflection mode. Dichroic mirrors (DM1 /DM2) select and direct wavelengths to CCD cameras in dark-field mode. Beam splitters (BS_1 and BS_2) with other two CCD cameras acquire bright-field images of the sample as shown on Fig.1. Broadened reference waves for interference producing are directed on CCD camera chip with small angle respect to object waves. Small CCD camera chip resolution necessitates mount values of the theses angles as small as possible.

Application of this method provides information on the type and arrangement of the external seed coat cells as well as the pattern of thickenings and pitting of the lateral and inner tangential cell walls of the seed coat. The seed coat is dark and opaque, but bleaching seeds allows a clear view of some surface features, particularly the contours and pitting of the seed coat epidermal cells [19]. Anticlinal walls medium to very deep and evenly thickened, with a narrow trough marking the wall junctures between cells, noticeable all around the edge of the cells, inner periclinal walls granulate or rugulose.

IV. Conclusion

Our study of the morphology of seeds of the 13 *O. cumana* populations, occurring in different geographical regions of RM, Romania, Spain and Ukraine, adds new data to the knowledge of seed micromorphology using the modern technology of DHM. The micrographs have illustrated that the broomrape seed coat is dark and opaque with a smooth membranous and granulated-rugulose outer periclinal walls, which indicate the provenance to the morphological seed type III as indicated in reference [10].

The practical application of a compact digital holographic microscopy module in our study showed some significant characteristics, like, the adaptability of DHM microscope, simplicity of sample preparation (no sectioning or staining is required, so that living cells and specimens can be observed in depth).

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