

TOOLS TO EVALUATE ESTROGENIC POTENCY OF DIETARY  
PHYTOESTROGENS: A CONSENSUS PAPER FROM  
THE EU THEMATIC NETWORK "PHYTOHEALTH" (QLKI-2002-2453)

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[Received May 5, 2006; Accepted October 15, 2006]

**ABSTRACT:** *Phytoestrogens are naturally occurring plant-derived polyphenols with estrogenic potency. They are ubiquitous in diet and therefore, generally consumed. Among Europeans, the diet is rich in multiple putative phytoestrogens including flavonoids, tannins, stilbenoids, and lignans. These compounds have been suggested to provide beneficial effects on multiple menopause-related conditions as well as on development of hormone-dependent cancers, which has increased the interest in products and foods with high phytoestrogen content. However, phytoestrogens may as well have adverse estrogenicity related effects similar to any estrogen. Therefore, the assessment of estrogenic potency of dietary compounds is of critical importance. Due to the complex nature of estrogenicity, no single comprehensive test approach is available. Instead, several in vitro and in vivo assays are applied to evaluate estrogenic potency. In vitro estrogen receptor (ER) binding assays provide information on the ability of the compound to I) interact with ERs, II) bind to estrogen responsive element on promoter of the target gene as ligand-ER complex, and III) interact between the co-activator and ERs in ligand-dependent manner. In addition, transactivation assays in cells screen for ligand-induced ER-mediated gene activation. Biochemical in vitro analysis can be used to test for possible effects on protein activities and E-screen assays to measure (anti)proliferative response in estrogen responsive cells. However, for assessment of estrogenicity in organs and tissues, in vivo approaches are essential. In females, the*

*uterotrophic assay is applicable for testing ER $\alpha$  agonistic and antagonistic dietary compounds in immature or adult ovariectomized animals. In addition, mammary gland targeted estrogenicity can be detected as stimulated ductal elongation and altered formation of terminal end buds in immature or peri-pubertal animals. In males, Hershberger assay in peri-pubertal castrated rats can be used to detect (anti)androgenic/ (anti)estrogenic responses in accessory sex glands and other hormone regulated tissues. In addition to these short-term assays, sub-acute and chronic reproductive toxicity assays as well as two-generation studies can be applied for phytoestrogens to confirm their safety in long-term use. For reliable assessment of estrogenicity of dietary phytoestrogens in vivo, special emphasis should be focused on selection of the basal diet, route and doses of administration, and possible metabolic differences between the species used and humans. In conclusion, further development and standardization of the estrogenicity test methods are needed for better interpretation of both the potential benefits and risks of increasing consumption of phytoestrogens from diets and supplements.*

**KEY WORDS:** Diet, Estrogenicity, Isoflavones, Lignans, Phytoestrogens

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## INTRODUCTION

Phytoestrogens are naturally occurring molecules of plant origin able to interact with estrogen receptors (ERs) or modulate estrogen action *in vivo*. These compounds are ubiquitous in diet and include polyphenols e.g. flavonoids, tannins, stilbenoids, and lignans. An earlier report of the Working group on Phytoestrogens and Health (COT Report, 2003) classified phytoestrogens according to their chemical structures into flavonoids (including isoflavones and prenylflavonoids), coumestans, and lignans. In this review, the main focus is on dietary isoflavones (e.g. genistein and daidzein) and lignans (e.g. matairesinol, and secoisolaricresinol) since their presence in diet and dietary exposure have been documented in different European countries (Keinan-Boker et al., 2002; van Erp-Baart et al., 2003; Valsta et al., 2003; Milder et al., 2004).

There is still an increasing interest in public and industry on plant-derived substances, especially phytoestrogens, acting as hormone mimics. Many of these compounds are marketed with health claims as dietary supplements or nutraceuticals. As a result, the nutritional and pharmaceutical use of dietary phytoestrogenic compounds has increased dramatically over the last decade. Number of reports suggests several health-promoting effects in the development of age-related diseases, such as atherosclerosis, hormone-dependent cancers, and osteoporosis (Branca and Lorenzetti, 2005; Adlercreutz et al., 2004). However, similar to any estrogens, dietary naturally occurring estrogenic compounds may as well have adverse effects and potentially act as endocrine disrupters. Therefore, the evaluation of estrogenic potency of various dietary phytoestrogens both as health promoting and endocrine disrupting compounds is of critical importance.

In this paper, we review most commonly used *in vitro* and *in vivo* test methods applied for assessing estrogenic potency of compounds. In addition, special attention is paid on factors involved in testing diet and food related compounds.

## DEFINITION OF ESTROGENS, PHYTOESTROGENS, AND SERMS

In this report, estrogens, both endogenous and exogenous, are defined as compounds whose biological actions are mediated by interaction with estrogen receptors (ERs). Many plants including dietary sources contain compounds with ER-interacting potency also defined as phytoestrogens. These are defined as *any plant substance or metabolite that induces biological responses in vertebrates and can mimic or modulate the actions on endogenous estrogens usually binding to the ERs* (COT Report, 2003).

Some phytoestrogens, such as isoflavones, have also been defined as Selective Estrogen Receptor Modulators (SERMs). These substances have estrogenic effects in selected target tissues, but no effects or antiestrogenic effects in others (Nilsson and Gustafsson, 2002; Meegan and Lloyd, 2003; Riggs and Hartmann, 2003; Cos et al., 2003). This definition was originally based on the preferential binding affinity of selected isoflavones to ER $\beta$  over ER $\alpha$  (Kuiper et al., 1998). Furthermore, dietary phytoestrogens may act as Selective Tissue Estrogenic Activity Regulators

(STEARs) (Smith and O'Malley, 2004). These compounds provide estrogenic activity via other routes than direct interaction with the receptors or are precursors for *in vivo* metabolism to produce compounds with endocrine activity (Smith and O'Malley, 2004). These definitions are often simplified and further interpreted to reflect the complex biological responses of SERMs and STEARs *in vivo*. As a result, conclusions on phytoestrogens beneficial effects, e.g. for menopausal health, are made based solely on *in vitro* data on binding of plant compounds to ER $\beta$  over ER $\alpha$ .

## ER Selective Ligands and *In Vivo* Knock Out (KO) Models as Tools to Assess ER-Mediated Functions

The availability of several compounds, which are selective ligands for ER $\alpha$  or ER $\beta$  has given novel possibilities to test the role of specific ER-ligands and SERMs such as isoflavones both *in vitro* and *in vivo*. *In vitro*, selective ligands all share at least 70-fold dissociation for either ER $\alpha$  over ER $\beta$  or vice versa (Meyers et al., 2001, Harrington et al., 2003). Among phytoestrogens, genistein is one of the compounds binding preferentially to ER $\beta$  (Kuiper et al., 1996), but the exact degree of dissociation has never been calculated for genistein or any other dietary compound. However, ligand binding activities of genistein extrapolated from the published data show 18, 19, or 31-fold preference for ER $\beta$  over ER $\alpha$  (Kuiper et al., 1998, Committee on Toxicity, 2003; Mueller et al. 2004). There are major differences in ER binding but the relative differences in stimulation of transcriptional activity are generally lower (Kuiper et al., 1998, Mueller et al., 2004). Isoflavones may also act differently from classical endogenous estrogens either by binding to other nuclear receptors (Giguere, 2002) or by recruiting different nuclear receptor co-regulators than endogenous estrogens (An et al., 2001), and may have selective ER $\beta$ -modulator activity and organ specific actions (Mäkelä et al., 1998). Therefore, the role of genistein as ER $\beta$ -interacting phytoestrogens is still under investigation. The *in vitro* models suitable for testing ER-mediated estrogenicity of isoflavones and other dietary compounds are further described in respective section later.

Uterotrophic screening of ER-selective compounds has revealed unique features of the ER-mediated estrogenicity. ER $\alpha$  ligands show significant uterotrophic activity while ER $\beta$  ligands are neither uterotrophic nor mammatrophic (Frasor et al., 2003; Harris et al., 2003; Hegele-Hartung et al., 2004; Hillisch et al., 2004). In addition, preliminary evidence obtained from immortalized normal HC11 mouse mammary gland cells showed ER $\alpha$  ligand-stimulated proliferation and expression of proliferation markers whereas a selective ER $\beta$  ligand inhibited proliferation and induced apoptosis (Helguero et al., 2005). Accordingly, in ER $\alpha$  expressing human breast cancer cells, expression of ER $\beta$  inhibited proliferation (Ström et al., 2004). These findings suggest that ER $\beta$  is associated with the antiproliferative response in estrogen-target tissues.

The development of knock-out mice models for ERs has revealed information crucial to understand the importance of ER-mediated estrogenicity. In knock-out (KO) mice, the phenotypes grossly matched the expression pattern of ER $\alpha$  and ER $\beta$

in different tissues (Korach et al., 1996; Krege et al., 1998; Couse et al., 2000; Dupont et al., 2000). In general, more drastic phenotypes and endocrine alterations were observed in ER $\alpha$ -KO mice while ER $\beta$ -KO mice have a relatively benign phenotype. ER $\alpha$ -KO mice were infertile, and had elevated serum LH, estradiol (E2) and testosterone levels, decreased bone density, and compromised development of the mammary gland (Korach et al., 1996; Krege et al., 1998; Couse and Korach, 1999; Couse et al., 2000; Dupont et al., 2000). Instead, ER $\beta$ -KO mice develop almost normal without apparent detrimental reproductive alterations (Dupont et al., 2000). However, some effects in follicular maturation and follicle ability in responding to specific stimuli have been reported (Couse et al., 2005; Emmen et al., 2005). These findings indicate that ER $\alpha$  and  $\beta$  have different functions *in vivo*. ER $\alpha$  is presumably associated with classical mechanisms of estrogen actions while ER $\beta$  has revealed new possible functions for estrogens (Harris et al., 2002; Koehler et al., 2005). This view is further supported by findings in gene array studies in uterus and bone of ER $\alpha$ -KO and ER $\beta$ -KO mice. Both studies report that the expression of the vast majority of genes is triggered through the ER $\alpha$ , whereas ER $\beta$  has to be regarded as a modulator of the magnitude of the ER $\alpha$  response (Hewitt et al., 2003; Lindberg et al., 2003).

The classical *in vivo* models most commonly used for testing estrogenicity of compounds are summarized in section 5 respective section later.

## INTAKE OF DIETARY PHYTOESTROGENS IN EUROPE

The limited information on phytoestrogen intake among Europeans has increased during this century (Tables 1 and 2). The food supply and composition differ geographically which may result in major differences in dietary phytoestrogen exposures. The exposure evaluation is mainly done by two methods: (1) by calculating phytoestrogen intake via diet, and (2) by determining phytoestrogen concentration in serum or urine. The majority of the estimates on dietary phytoestrogen intake rely on food consumption data collected with food records, food frequency questionnaires or dietary recalls, and international or national food composition databases. Earlier intake estimates have been mainly based on frequent intake of certain food items, i.e. known sources of phytoestrogens, for which phytoestrogen content have been available

(Keinan-Boker et al., 2002). More detailed European databases that contain information on phytoestrogens (lignans and/or isoflavones) include the Finnish National Food Composition Database (Fineli) (Valsta et al., 2003) and the Vegetal Estrogens in Nutrition and the Skeleton (VENUS) database (Kiely et al., 2003). The VENUS database has been used at European level (Erp-Baart et al., 2003) and parts of it at regional level, e.g., in the UK (Grace et al., 2004). Also other databases have been compiled. These are based on regional needs and include newly found phytoestrogens (plant lignans), e.g. in the Netherlands (Milder et al., 2005) and in Sweden (Hedelin et al., 2006).

**TABLE 1. Isoflavone intake of adults, soy-consumers, vegetarians and vegans in the USA and in the European countries.**

COUNTRY	N	SUBJECTS	INTAKE, MG	REFERENCE
USA	447	women, 50-79 y	2.87	Horn-Ross et al., 2000
USA	964	postmenopausal women	0.76	De Kleijn et al., 2001
USA	2882	women, 35-79 y	3.3	Horn-Ross et al., 2001
USA	111526	women, 21-103 y	1.78	Horn-Ross et al., 2002
10 European countries	35 955	whole population, 35-74 y	< 2	Keinan-Boker et al., 2002
UK	335	whole population	0.72	Van Erp-Baart et al., 2003
UK	333	women, 45-75 y	0.54	Grace et al., 2006
UK	15	soy-consumers	3.2	Van Erp-Baart et al., 2003
UK	35	vegetarians	12	Clarke et al., 2003
Ireland	1379	whole population	0.73	Van Erp-Baart et al., 2003
Ireland	42	soy-consumers	6.0	Van Erp-Baart et al., 2003
Italy	1513	whole population	0.56	Van Erp-Baart et al., 2003
NL	4085	whole population	0.91	Van Erp-Baart et al., 2003
NL	85	soy-consumers	11.1	Van Erp-Baart et al., 2003
Finland	2862	whole population, 25-64 y	0.79	Valsta et al., 2003
Sweden	1130	population based controls	0.34	Hedelin et al., 2006
Greece		weekly menu of the traditional Greek diet	1.3	Vasilopoulou et al., 2005

**TABLE 2. Lignan intakes of adults in the USA and in the European countries**

COUNTRY	N	SUBJECTS	INTAKE, MG	REFERENCE
USA	107	men, mean age 60.6 y	0.53 <sup>1)</sup>	Strom et al., 1999
USA	447	women, 50-79 y	0.18	Horn-Ross et al., 2000
USA	1610	women, 35-79 y	-0.15 <sup>1)</sup>	Horn-Ross et al., 2001
USA	964	postmenopausal women	0.64	De Kleijn et al., 2001
USA	558	women, 20-74 y	-0.10 <sup>1)</sup>	Horn Ross et al., 2002a
USA	111526	women 21-103 y	0.11	Horn-Ross et al., 2002b
USA	136	men, 18-55 y	-1.36 <sup>2)</sup>	Walcott et al., 2002
USA	470	women, 35-79 y	0.17 <sup>1)</sup>	Horn-Ross et al., 2003
NL	17140	women, 50-69 y	1.1	Keinan-Boker et al., 2002
Finland	2862	whole population, 25-64 y	0.43	Valsta et al., 2003
Finland	2852	whole population, 25-64 y, without "linseed eaters"	0.32	Kilkinen et al., 2003
Germany	666	women, < 50 y	0.56	Linseisen et al., 2004
NL	4660	whole population, 19-97 y	1.24 <sup>4)</sup>	Milder et al., 2005 a
Sweden	1130	population based controls	4.86 <sup>5)</sup>	Hedelin et al., 2006

1) Median intake

2) Estimated from median energy (1941 kcal) and lignan (698  $\mu$ g/1000 kcal) intakes

3) Seco+Mat

4) Seco+Mat+Lari+Pino

5) Seco+Mat intake of all foods, Lari+Pino+Syr+Med intakes from bread and cereal products included

The average isoflavone intake from European diet varies from about 0.01 mg/day to about 1 mg/day. This is somewhat less than from the average U.S. diet (Table 1). The isoflavone intake is clearly higher among soy-consumers, vegetarian, and vegans in Europe ranging from about 3 mg/day up to > 10 mg/day (Table 1). Among infants the average isoflavone intake has been estimated to differ from 3 mg/kg/day up to 13 mg/kg/day (Setchell et al., 1998; Rupp et al., 2000). The sources of isoflavones in the European diet are mainly soy flour, soy beans, and soy-derived ingredients in processed foods, e.g. in meat products and bread (Keinan-Boker et al., 2002; Valsta et al., 2003).

In several European countries the mean intake of lignans, estimated as a sum of plant lignans secoisolaricinol (SECO) and matairesinol (MAT) has been shown to be around or below 1 mg/day (Keinan-Boker et al., 2002; Kilkkinen et al., 2003; Linseisen et al., 2004). The previous European estimates of lignan intakes do not differ significantly from the lignan intake estimates in the U.S. (Table 2). However, recent reports on the presence of new plant lignan precursors for mammalian lignan production in foods, have changed the view of lignan intake estimates (Heinonen et al., 2001; Milder et al., 2005b). Food composition analyses of lariciresinol (LARI) and pinoresinol (PINO) demonstrated that in many food items these lignans are dominating and may contribute to about 75% of the total lignan intake (Milder et al., 2005a). Inclusion of additional dietary lignans such as syringaresinol (SYR) and medioresinol (MED) have resulted in approximately four-fold intake estimates (Hedelin et al., 2006). This suggests that the former lignan intake estimates using only SECO and MAT have been clear underestimates. The major sources of lignans in Europe are cereals, coffee, tea, fruit, berries, vegetables, and also alcoholic beverages (Valsta et al., 2003; Kilkkinen et al., 2003; Milder et al., 2005a-b). One of the richest food source of lignans is linseed (Valsta et al., 2003; Milder et al., 2005b) and including linseed consumers in the intake calculations increases significantly the average intake values (Kilkkinen et al., 2003; Valsta et al., 2003).

When the intake of isoflavones and lignans is estimated, the quality of the food composition database is important. This is most critical in case of foods containing ingredients with concentrated amounts of phytoestrogens, e.g. soy as such or soy protein as a source of isoflavones or linseed in different forms as a source of lignans. In addition, the reliable phytoestrogen values of foods consumed regularly or in large quantities are of major importance when intake estimates are produced (Kiely et al., 2003; Valsta et al., 2003). Therefore, food composition databases with local representative foods should be preferably used especially for national intake estimates. Because of the increasing complexity of the food supply, there are major challenges in collecting reliable food consumption data for phytoestrogen intake estimates. Additionally, it is important to recognize that among those population groups consuming foods with concentrated phytoestrogen preparations or phytoestrogen-supplements, normal diet plays a minor role in phytoestrogen intake.

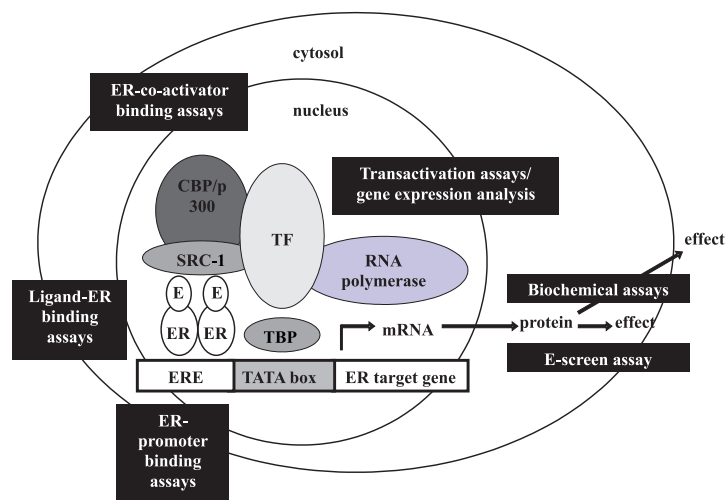
## IN VITRO ASSAYS FOR TESTING ESTROGENICITY

The classical (anti)estrogenic activity of any compounds (plant-derived or man-made ones) is determined by its ability to bind to the

estrogen receptors, ER $\alpha$  and ER $\beta$ . The ability to interact with these receptors results in potential to act as “endocrine disruptors”. Several attempts have been made by the scientific community to provide *in vivo* and *in vitro* assays, suitable to assess the (anti)estrogenicity of natural and synthetic compounds. Available *in vitro* methods to screen and assess (anti)estrogenicity are based on the measurements of different steps of 17 $\beta$ -estradiol (E2) signaling mediated by ERs. Figure 1 shows the molecular mechanism of the classical ERs-mediated actions and provides the basis for the set up of a series of assays, such as a) ligand-ER binding; b) ER-promoter binding; c) the ER-co-activator association; d) the transactivation (of gene expression); e) biochemical; and f) E-screen assays.

### FIGURE 1. Summary of different steps of ER-mediated signaling are presented. Each step provides an assay point for the assessment of (anti)estrogenicity of a given compound.

E: ER-ligand; ERE: estrogen responsive element; CBP/p300 and SRC-1: co-activator proteins; TBP: TATA binding protein; TF: transcription factor. (Modified from Mueller, 2002).



#### (a) Ligand-ER Binding Assays

The ligand-ER binding assay measures the ability of a compound to interact with ERs. Binding to ERs is the first step of the classical ER signaling cascade and also a “pre-requisite” to define a molecule as estrogen-like. The ligand-ER binding assay is performed by using radio-labeled E2 as competitor of the compound to be tested at ER binding sites (Korach, 1979). Hence, the assay provides an evaluation of the binding affinity of each test compound toward ER $\alpha$  or ER $\beta$  relative to E2 (Kuiper et al., 1997; Kuiper et al., 1998).

The introduction of fluorescent probes, has made possible the measurement of relative binding affinities using fluorescence polarization by the utilization of fluorescein-labeled E2 (Bolger et al., 1998). The radio-labeled version of the ligand-ER binding assay has the advantage to allow the measurements of the binding activity of purified compounds on both crude protein extracts and intact living cells expressing ERs. Fluorescein-labeled approach provides reliable results only with purified ERs. This limitation is

due to the high background of the fluorescein labeling in crude protein extracts (Nagel et al., 1998). On the other hand, fluorescence polarization made possible high-throughput ER-binding screening of hundreds of compounds with a very favorable time-cost ratio.

Together with the assessment of the relative binding affinity, the assessment of the ligand-ER binding activity can provide the basis for the measurement of another binding parameter: the ER conformational change after ligand binding. On the basis of the suggestion that there might be a relationship between ER structure and activity it has been proposed that different ER modulators may induce conformational changes in the receptor resulting in a specific biological activity. Paige and coworkers (1999) observed that different ER ligands, known to produce distinct biological effects, induce distinct conformational changes in the receptors, providing a strong correlation between ER conformation and biological activity. The same authors have demonstrated that the ability of some peptides to discriminate between different ER $\alpha$  and ER $\beta$  ligand complexes suggests that the biological effects of ER agonists and antagonists acting through these receptors are likely to be different.

This possibility has been used owing to directly link conformational changes to agonist/antagonist properties toward ER $\alpha$  and ER $\beta$  of compounds already known to bind ERs (Wijayarathne et al., 1999; Norris et al., 1999).

### (b) ER-Promoter Binding Assays

Once ligand-ER binding has occurred, the binding of ER-dimers to an estrogen responsive element (ERE) on the promoter of its target genes is the second step in the classic ligand-ER signaling pathway. The ER-promoter binding assay measures the ligand-dependent binding of an ER to a radio-labeled or fluorescein-labeled ERE. Notably, promoters of estrogen-responsive genes differ in their EREs (Nardulli et al., 1996; Dana et al., 1994) and ER $\alpha$  and ER $\beta$  differ in their binding ability to different EREs (Hyder, 1999; Nikov et al., 2000). Even though in theory elegant and informative, this assay often failed to detect differences in the affinities of each tested ternary complexes, due to the high constitutive binding of ERs to ERE (Nikov et al., 2000; Boyer et al., 2000; Curtis and Korach, 1990).

### (c) ER-Co-Activator Binding Assays

Since ER binding to the promoter depends on the presence of a number of different co-activators to be assembled on an active transcriptional complex, a different approach to detect a ligand-induced conformational change in the ER (Shiau et al., 1998) is based on the measurement of protein-protein interactions. One of the most utilized is the "GST (glutathione S-transferase) pull-down assay" (Nishikawa et al., 1999), based on a fusion protein of GST and the ER of interest (GST-ER). The GST-ER fusion protein is incubated with a radio-labeled co-activator in the presence of an ER ligand. The protein complex is purified (by a glutathione affinity chromatography), separated on acryl amide gel (by SDS-PAGE) and the binding of the co-activator to the GST-ER is detected and quantified by autoradiography. The

interaction between the co-activator and the ER is associated to the characteristics of the ligand and proportional to its response. These associations allow the determination of the potency of a compound in triggering the binding of ER $\alpha$  and ER $\beta$  with a co-activator (Onate et al., 1995; Nishikawa et al., 1999; Kraichely et al., 2000; Routledge et al., 2000).

Ligand-dependent ER-co-activator binding is also detectable utilizing fluorescent-labeled co-activators and ER (or ER-ligands) coupled with fluorescence resonance energy transfer (FRET) (Zhou et al., 1998; Gee et al., 1999; Sun et al., 1999). ER-coactivator binding measurement directly correlates with the amount of energy transfer from the fluorescent group of ER or ER-ligand to the co-activator, thus allowing a dose-dependent quantification and the (anti)estrogenic evaluation as well.

An alternative approach that can be utilized to detect ER-co-activators binding in living cells is the yeast "two-hybrid" assay. This system detects protein-protein interactions through the ability to reconstitute the activity of two separated domain of a yeast transcription factor following the activation of the yeast  $\beta$ -galactosidase reporter gene by chemiluminescence. This approach requires the introduction in yeast cells of two expression plasmids carrying, respectively, the ER of interest and the putative co-activator fused to two different domains of the yeast transcription factor GAL4. Once ER-co-activator binding take place, GAL4 domains reconstituted the whole enzyme able to recognize a chromosomal integrated  $\beta$ -galactosidase gene. The promoter of the reporter gene carries one (or more) ERE(s). Finally, the integrated reporter gene is activated only when, and if, the binding between ER and the co-activator takes place.

A limiting factor in the employment of this system is due to the different set of post-translational modifications in yeast in comparison with mammalian cells. However, the transformation of a suitable mammalian cell type with a third expression plasmid carrying the yeast  $\beta$ -galactosidase as a reporter gene, makes possible to perform a "two-hybrid" assay in the proper cell context, overcoming any possible interference with different post-translational modifications (Nishikawa et al., 1999).

### (d) Transactivation Assays and Gene Expression Analysis

Ligand-induced ER-mediated gene activation is based on the co-transfection of ER $\alpha$  (or ER $\beta$ ) cDNA together with a reporter gene containing one or more EREs (McDonnell et al., 1995; Shelby et al., 1996; McDonnell, 1999). The transactivation assay is performed in living cells (yeast or mammalian cells) devoid of endogenous ER, transiently co-transfecting them with expression plasmids carrying: a) the ER of interest; and b) a reporter gene (either chloramphenicol acetyltransferase/CAT or luciferase cDNA), whose activity depends on its promoter region where one or more EREs (or even the entire promoter region of any ER target gene) can be placed. The induction of the reporter gene depends on the addition of ER ligands and the induced dose-dependent transcription can be easily measured.

The transient transactivation assay is very sensitive and versatile and it is widely considered the method of choice to analyze both weak and potent estrogens and estrogen-like (including phyto-

and xeno-estrogens) molecules. This method allows the assessment of (anti)estrogenicity of single molecules, or complex mixtures, and also a simultaneous fast high-throughput analysis of many compounds (and their combinations) (McDonnell, 1999; Mueller 2002). The limitation of the assay is due to the artificial induction of ER gene expression in cells that are devoid of ER in their wild genotype. This “forced expression” might be not reflecting a real physiological response. This concern has been overcome by performing the assay in cell lines having their own endogenous ER activity i.e., MCF-7, expressing mainly ER $\alpha$  (?Mueller and Korach, 2001), and only transfecting the ERE-reporter gene construct.

In cell lines characterized by an endogenous ERs expression, the downstream expression of ER target genes might also be monitored without the need of an artificial reporter gene transfection. This approach is the closest to the physiological cellular response to treatment with any ER ligand, and it allows to discriminate specific features between different tissues and cell types. Moreover, the utilization of cells owing ERs enables the detection of different ER ligands activity in different contexts (i.e., the estrogenicity of tamoxifen in breast and uterus and its anti-estrogenicity in bone). Specific mRNA expression (by real time RT-PCR, Northern blot) and protein expression (by Western blot) are usually suitable end-points. Notably, even though different genes or proteins can be monitored at the same time, the same genes or proteins might, or might not, be the appropriate marker to establish the (anti)estrogenic response associated with specific features of different tissue and cell lines. Furthermore, serial measurements following treatment are always necessary to keep into account the time course of gene expression. Recently, the introduction of “high through put” techniques at transcriptomic and proteomics level, have allowed the detection of the differential expression of thousands genes either at the level of mRNA or its gene product, the protein. These approaches, must be considered valuable tools for extensive insights on the molecular mechanisms and on the tissue-specific effects of (anti)estrogenic molecules, are not easily suitable to screen a large number of different molecules with a putative ER-mediated effect (Mueller, 2002).

#### (e) Biochemical Assays

Downstream the analysis of gene or protein expression, the monitoring of protein activities is

an expedient key to study *in vitro* the final effect of (anti)estrogenic molecules in different cell types. Different types of this approach have been used to address: i) the regulation of alkaline phosphatase activity in osteoblast and endometrial (Ishikawa) cell lines (Kanno et al., 2004; Markiewicz et al., 1993); ii) activity and secretion of TRAP (tartrate-resistant acid phosphatase) in osteoclast cell lines, to monitor the modulation of osteoclastogenesis (Kanno et al., 2004); iii) the inhibition of PSA (prostate specific antigen) secretion in prostate cancer cell lines to detect decrease or enhanced cell proliferation (Rosenberg Zand et al., 2002); iv) the interference on the activity of enzymes involved in steroidogenic metabolisms and steroid biosynthesis (e.g. aromatase inhibition) to reveal an (anti)estrogenic role of certain compounds on the estrogen biosynthesis (Pelissero et al., 1996; Gray et al., 1988)

#### (f) E-screen Assay

The most widely used assay to measure (anti)estrogenic response is based on the measurement of cell proliferation after treatments with increasing concentrations of a specific molecule (Soto et al., 1995). Cell proliferation assay is performed in cells expressing functional, endogenous ERs and it is often claimed to be a reliable tool to assess the physiological response to an (anti)estrogenic action (Soto et al., 1998). The main limitation of the E-screen assay is the lack of the ability to discriminate between the role of ER $\alpha$  and ER $\beta$ .

**TABLE 3. Summary of *in vitro* assays available to assess (anti)estrogenic activities. (adapted from COT Report, 2003)**

IN VITRO ASSAY	MEASURED OUTPUT	ADVANTAGES	DISADVANTAGES
Ligand-ER binding	Both ER $\alpha$ and ER $\beta$ binding affinity data	Easy to perform; high-throughput	No ER activation data
ER-promoter binding	Both ER $\alpha$ and ER $\beta$ binding affinity data to different EREs	Easy to perform; high-throughput	No ER activation data
ER-coactivator binding	Both ER $\alpha$ and ER $\beta$ ligand-dependent binding to co-activators	Dissection of molecular interactions; discriminate estrogenic/anti-estrogenic action	No ER activation data; Artificial system
Transactivation	Both ER $\alpha$ and ER $\beta$ data on reporter gene activation	Simple; high-through put; discriminate estrogenic/ anti-estrogenic action	Artificial system
Gene expression analysis	Both ER $\alpha$ and ER $\beta$ regulated gene and protein expression	Physiological output; discriminate estrogenic /anti-estrogenic action	Low throughput
Biochemical assays	Steroidogenic enzymes activities; or estrogen biosynthesis analysis; or ER-dependent activities of target genes	Physiological output; discriminate estrogenic/anti-estrogenic action	Only cell lines with expressed molecular markers
E-screen	ER $\alpha$ -dependent cell proliferation	Physiological endpoint; discriminate estrogenic/ anti-estrogenic action	No defined ER expression; no ER $\beta$ -dependent cell proliferation assay yet available

**(g) Final remarks on *in vitro* assays**

All the different *in vitro* tools that can be used to characterize the (anti)estrogenic activity of phytoestrogens described above are summarized in Table 3. Indeed, many phytoestrogens have been analyzed only in some of these assays, and in some cases their activity has been calculated as relative either to E2 and diethylstilbestrol (DES) (reviewed in Lorenzetti, 2005).

Previously, only four different *in vitro* assays (the most widely used) were listed as suitable and reliable tests to assess the estrogenic potency of phytoestrogens, namely: the ligand-binding assay, the E-screen, the transactivation assay, and the gene expression analysis (COT Report, 2003). Importantly, the result of the estrogenic potencies of some phytoestrogens in the different *in vitro* assays ranking them as related to E2 were compared. It was also pointed out that a real standardization of different techniques has never been done and, probably, an adequate and full assessment of estrogenic potency of phytoestrogens would require the validation of a set of *in vitro* and *in vivo* assays.

Finally, some isoflavones and flavonoids (Suetsugi et al., 2003), as well as different xeno-estrogens (reviewed in Ariazi and Jordan, 2006), have been reported to bind and possibly act either as agonists or antagonist to estrogen-related receptors (ERRs) (Horard and Vanacker, 2003). Even though estrogens, or any other natural ligand have not yet been shown to bind ERRs (Ariazi and Jordan, 2006), these observations raise the question whether the already known, and possibly underestimated, interplay between ERs and ERRs transcriptional activities (reviewed in Giguere, 2002) should add another level of complexity in testing (anti)estrogenicity.

**TESTING ESTROGENICITY *IN VIVO***

Laboratory rats and mice are species most commonly used in estrogenicity assays *in vivo*. Rodents have been widely used in biological research, and therefore their anatomy, physiology, pathology, and genetic background are well characterized. Furthermore, they are easy to breed, and have short generation time, which allows investigation of the potential effects in all developmental stages of living organism (*in utero*, during lactation, pre- or peri-pubertally, or in adulthood), or even prior to mating (parent generation).

The OECD test guidelines for chemicals comprise many methods focusing on indications for endocrine effects. The one- or two-generation test (OECD guidelines 415 and 416) may be considered as the most comprehensive methods to investigate endocrine-related tissues and the reproductive functions. Especially the two-generation test ideally covers a broad array of endpoints for screening of putative estrogenicity and also other endocrine specific mechanisms. However, for screening purposes of estrogenicity, acute or sub-acute tests are of advantage. OECD test strategy for endocrine active compounds comprise a battery of short-term assays including uterotrophic assay for (anti)estrogenicity, Hershberger assay for (anti)androgenicity, and the so-called “enhanced” sub-acute test (TG 407) for (anti)estrogenicity, (anti)androgenicity, and (anti)thyroid effects in context to general toxicity (Gelbke et al., 2004). These assays are also applicable for testing dietary compounds either as single ingredients, mixtures, or as a part of diet (see section 6.3).

**(a) Validated *In Vivo* Estrogenicity Assays****(i) Uterotrophic Assay**

The most common short-term *in vivo* assay for (anti)estrogenicity is the uterine growth test, suitable for screening ER $\alpha$  agonists and antagonists. Either immature intact or adult ovariectomized female rats or mice are used (Odum et al., 1997; Kang et al., 2000; Laws et al., 2000; Cotroneo et al., 2001; Newbold et al., 2001; Kanno et al., 2003). The test compounds are administered either subcutaneously or orally for a period of 3–7 days. The primary end-point is uterine wet weight. Increase in uterine weight indicates an estrogenic activity of the test compound, while an antiestrogenic compound will diminish the estrogen dependent uterine weight increase in intact immature or ovariectomized adult females. In order to enhance the sensitivity of the bioassay, additional morphological and biochemical end-points in the uterus may be included in the test protocol. These include determination of uterine epithelial cell height, uterine gland formation, cell proliferation, rat uterine gene expression e.g. complement C3, progesterone receptor, clusterin, and the production of the estrogen-inducible protein, lactoferrin in the uterine epithelial cells (Ashby, 2001; Diel et al., 2001; Newbold et al., 2001; Jefferson et al., 2002; Diel et al., 2004).

**(ii) Hershberger Assay**

Hershberger assay is a short-term *in vivo* screening assay for (anti)androgenicity. Several variations of the test protocol exist. The test is usually performed on peri-pubertal male castrated rats (Ashby et al., 2004; Suzuki et al., 2005). For assessment of androgenicity, test compounds are administered to castrated males, while for anti-androgenicity, test compounds are given to castrated, testosterone-treated animals. The test compounds are administered either subcutaneously or orally for a period of 10 days (Stroheker et al., 2003; Ashby et al., 2004; Yamada et al., 2004) but shorter (3–7 days) dosing periods have also been used (Yamada et al., 2004). The measured end-points are the weights of androgen dependent accessory sex glands. The mandatory organs for weight record are the ventral prostate, seminal vesicles together with coagulating glands, glands penis, Cowper’s glands, and *levator ani* and *bulbocavernous* muscles. Furthermore, optional end-points like the weights of liver, adrenal glands, kidneys, and blood levels of serum luteinizing hormone and testosterone may be included (Gray et al., 2002). Androgenic compounds increase the weight of androgen dependent tissues, while anti-androgenic compounds diminish this weight in testosterone treated immature castrate rats. As an alternative to castrated male rats in the Hershberger anti-androgen assay, testosterone-stimulated weanlings have been proposed (Ashby et al., 2004).

**(b) Indication of Estrogenicity in Other Female Organs *In Vivo***

In addition to validated uterotrophic assay the estrogenicity of dietary compounds can be evaluated in other estrogen responsive target tissues in female organism. Mammary gland as a hormone target organ can be used to investigate specific biological endpoints for estrogenicity. During isometric growth phase in immature

animals, mammary gland ducts elongate and branch slowly. At the onset of puberty, however, the ducts enter an allometric growth phase when the mammary gland is growing more rapidly than the whole body until the ducts have reached the periphery of the mammary fat pad (Richert et al., 2000). At this growth phase, ductal elongation is stimulated mainly by estrogens, while ductal branching is mediated by both estrogens and progesterones (Hovey et al., 2002). Therefore, in immature female rodents estrogenicity of the test compound can be monitored as enhanced ductal elongation and number of terminal end buds (TEBs). TEBs are located at the tips of the ducts with high proliferative activity (Richert et al., 2000) and are the main targets for ductal mammary carcinogenesis in rodents (Welsch et al., 1985; Russo et al., 2001). Reducing number of TEBs has been used as an indicator for (anti)estrogenicity leading to decreased mammary tumor multiplicity in carcinogen induced rats (Russo and Russo, 1996; Hilakivi-Clarke et al., 2001). In addition to enhanced mammary gland development, the exposure to estrogens (endogenous or exogenous) of immature animals advances the onset of puberty. In rats and mice this can be monitored as earlier vaginal opening and first estrus than in non-exposed animals (Ashby et al., 2000).

## TESTING ESTROGENICITY OF FOOD AND DIETARY COMPOUNDS *IN VIVO*

Phytoestrogens have been suggested to have beneficial effects on menopause-related symptoms such as hot flashes, osteoporosis, and age related development of atherosclerosis. Also their potential benefit in prevention of certain cancers is widely discussed in the literature. Therefore, there is an increased interest to develop foods and supplements with high phytoestrogen content.

When phytoestrogens are consumed as a part of diet, people are exposed to these compounds at different developmental stages and for long time periods. This gives additional challenge for testing of their putative estrogenicity *in vivo*. Moreover, if the consumption of phytoestrogens will be increased via human diet with regard to their possible health promotion, it is of crucial issue to verify their safety. In uterotrophic assays, endogenous (E2), synthetic (DES) and phytoestrogen (genistein) have been shown to induce similar pattern of gene transcription responses in immature mouse uterus suggesting intrinsic similarities between estrogens of different origin (Moggs et al., 2004). The naturally occurring dietary phytoestrogens may have the estrogenic potency to cause severe endocrine disruption such as infertility in domestic animals (Messina, 2002). This should be considered as an indication of the importance of evaluating the safety of increased dietary consumption of these putatively endocrine active compounds.

### (a) Selection of Diet Formulation for Estrogenicity Testing *In Vivo*

For testing the estrogenic potency of dietary compounds *in vivo*, selection of basal diet needs special emphasis. Several diet formulations are available for laboratory animals. The most

common types of laboratory animal diets are based on natural plant ingredients and are fixed open-formula diets. In these standard chow diets, soy bean meal and grains are commonly used (Nutrient Requirements of Laboratory Animals, 1995). Both soybean and grains are rich sources of phytoestrogens, isoflavones and lignans, respectively (Thigpen et al., 1999; Brown and Setchell, 2001; Degen et al., 2002; Saarinen et al., 2002). Therefore, it is recommended to avoid laboratory chows based on crude plant components in studies designed to test the estrogenic potency of dietary compounds. The phytoestrogenic compounds present in chow diets may significantly affect experimental outcome (Thigpen et al., 1987; Thigpen et al., 2004). Instead, purified semi-synthetic or synthetic phytoestrogen free diets are recommended. These diets are composed of refined, invariant, and restricted set of ingredients, which offer less variable and more easily controlled experimental conditions. Additionally, the use of purified or synthetic diets instead of natural ingredient chows, gives more precise control over the metabolizable energy, dietary composition of nutrient, and provides better repeatable experimental conditions (Ritskes-Hoitinga, 2001; Thigpen et al., 2002; Odum et al., 2004). However, it is still an open question if the experimental diet should be a standard for the chosen species or should mimic human Western diet with regard to fat, fibre, and calcium content.

The dietary compounds can be tested for estrogenicity as isolated chemicals (an isoflavone or a lignan) or as a part of dietary source (e.g. a soy bean protein, soy bean meal preparation, or flaxseed). The use of isolated compounds allows the determination of dose levels required for specified *in vivo* response and putative synergistic, additive, or inhibitory effects. However, dietary compounds are not consumed as isolated compounds but as a mixture with complex food matrices, which may affect their bioavailability, rate of metabolism, and biological responses *in vivo*. The non-estrogenic compounds present in plant sources or the food matrix may interact with estrogenic compounds and either potentiate, or suppress their activity and bioavailability. Therefore, in addition to testing dietary compounds in a purified form alone or as mixtures, their putative estrogenicity should also be assessed as a part of the food matrix used in human diet.

### (b) Recommended Route of Administration

When testing the estrogenic potency of dietary compound the route of administration is important. When isolated compounds from dietary origin are investigated those can be administered to animals by mixing with basal diet, oral gavage, *s.c.* or *i.p.* injections, or as *s.c.* implant. Administration via diet and by oral gavage mimics the natural route of exposure to dietary compounds and is therefore recommended.

In many cases, however, the dietary compounds (e.g. phytoestrogens) are further metabolized *in vivo* to compounds with potentially more biologically active. For example, daidzein present in soy is metabolized to equol and *O*-desmethylangolensin and some plant lignans such as secoisolariciresinol and matairesinol are metabolized to enterolactone by the intestinal microbiota (Rowland et al., 2003; Lampe et al., 2003). The metabolic activity

varies in individual animals. The serum phytoestrogen metabolite concentrations may vary significantly between equally aged animals from the same strain and gender after equal exposure dose, time, and duration. Therefore, when the dietary phytoestrogen metabolite is the main target of interest, the metabolite can be administered as such to minimize this inter-individual variation in metabolism. Depending on the chemical stability and pharmacokinetics of the test compound, it can be administered via diet or injected s.c. or i.p. However, in multiple dose or long-term exposure studies dietary administration is recommended.

### **(c) Applicability of the Standardized *In Vivo* Estrogenicity Assays for Investigation of Diet or Dietary Compounds**

The validated short-term *in vivo* tests, which can be applied to investigate potential estrogenicity of dietary compounds, are uterotrophic and Hershberger assays which provide an answer on possible interference of the test compounds on the function of endocrine system. Both tests are broadly used to test chemicals for potential endocrine activity and are currently being internationally evaluated under the OECD Test Guideline program (Gelbke et al., 2004; Yamada et al., 2004). These tests provide information, whether a compound or diet has (anti)estrogenic potency in uterus or (anti)androgenic potency in androgen sensitive target tissues. Accordingly, *in vivo* assays in other hormone-sensitive tissues (such as mammary gland) give information regarding possible (anti)estrogenicity in the investigated target tissue.

When dietary compound(s) or a certain type of diet is found to affect these targets, the outcome can not be automatically regarded as “beneficial”. Rather, responses in the uterotrophic and the Hershberger assays give an indication for need of additional studies. These may include test for endocrine effects and/or reproductive toxicity studies for which there are OECD guidelines, e.g. sub-acute to chronic exposure involving the examination of endocrine-related target tissues and the reproductive function or performance. No response in uterotrophic assay in a thorough dose-response study indicates that the test compound is not estrogenic in uterus *in vivo*. Equally, no response in the Hershberger assay indicates that the test compound is neither androgenic nor a 5- $\alpha$  reductase inhibitor in screened androgen-dependent tissues *in vivo*. However, these assays screen the (anti)estrogenicity and (anti)androgenicity in a limited number of tissues, and the test compounds found negative in these assays may still have endocrine activity in other target tissues. Additionally, it has to be remembered, that the rodent models have some limitations in predicting the (anti)estrogenic effects in humans e.g. in rats and mice the extragonadal synthesis of androgens as well as aromatase activity are very low. Therefore, demonstrating the effects on aromatase in these animal models is challenging.

Uterotrophic or Hershberger assays do not provide information on the possible (anti)estrogenic/(anti)androgenic effects in adult animals or in other hormone target tissues. In addition, questions that need to be investigated are whether increasing intake of dietary estrogenic compounds could alter production of steroids, and whether any alteration could result in disruption of

imprinting of subsequent sexual behavior in populations with traditionally low intake of these compounds. For this purpose, OECD guidelines 415 and 416 covering both one and two generation studies may give additional information. These guidelines may be considered as most comprehensive methods to investigate endocrine-related tissues and reproductive functions. Especially the two-generation test ideally covers a broad array of endpoints, not related to only one specific endocrine mechanism. Furthermore, it is worth considering including observations on development of the offspring in studies designed to investigate potential beneficial anti-cancer or anti-atherosclerotic effects of dietary estrogenic compounds in specific rodent models in experimental set-up with exposure at different developmental stages. However, these tests use large numbers of animals, and are costly and time consuming.

Finally, it is important to recognize, that the *in vivo* estrogenicity assays described have been developed primarily for testing reproductive toxicity and developmental defects. Therefore, they do not provide information on possible beneficial effects of dietary compounds with regards to cancer, atherosclerosis, osteoporosis, or menopausal symptoms relief. Therefore, the possible effects of dietary compounds in these areas of human health should be tested in animal models developed specifically for that purpose.

## **CONCLUSIONS AND FUTURE PERSPECTIVES**

Diet contains multiple compounds that may have estrogenic potency. These compounds, phytoestrogens, have gained a lot of interest as potentially health promoting agents. Generally, phytoestrogens as natural compounds in plant are considered as a “safer” alternative for the use of synthetic estrogens especially among aging Western populations. However, this issue is controversial, because similar to endogenous or synthetic estrogens natural compounds may as well promote adverse effects.

Since the approval of soy protein associated health claims by FDA (USA, Food and Drug Administration, 1999), the general interest to isoflavones as well as other phytoestrogens such as dietary lignans has increased significantly. Currently, many food companies are supplementing their products with ingredients rich in these compounds, and their consumption as a part of daily diet or as supplements is likely to further increase in future. Therefore, it is important to verify the safety of increased intakes among populations with traditionally low intakes of phytoestrogens. To be able to do reliable assessment of phytoestrogen's estrogenic potency and to set up experimental conditions relevant for human exposures, information on exposure to these compounds in different populations is crucial. The food supply and composition differ geographically and the food composition databases with values for local representative foods should be preferred. Moreover, it is important to specify the groups consuming diet especially high in phytoestrogens and to have reliable estimates of their intakes as well.

For evaluation of estrogenic potency of dietary phytoestrogens no single simple test is available that would answer a question, whether the compound is estrogenic or not. Instead, multiple

standardized *in vitro* tests targeted to describe different mechanisms of ER-mediated estrogenicity are available for screening the putative estrogenic activity of the compounds. Most of the data on phytoestrogens available so far have been obtained either with the E-screen assay (no ER $\alpha$  and ER $\beta$  discrimination) or with ligand-binding and transactivation assays (ER $\alpha$  and ER $\beta$  comparison usually available). However, the *in vitro* tools lack the capability to give estimates of the whole physiological effect of ER-mediated estrogenicity. Therefore, they can not fully predict the responses *in vivo*. At present, the use of *in vivo* models is unavoidable for reliable assessment of possible estrogenicity in multiple target organs and tissues. In addition, estrogenicity in one target organ/tissue does not necessarily indicate that the effects will occur in other organs/tissue. Thus, the assays must be optimized for the specific target organ/tissue of interest. Moreover, when setting dietary doses for the experiment, the differences in metabolism between the chosen species and humans should be taken into account.

When testing the putative estrogenicity of dietary phytoestrogens in animal models, the route of administration should mimic that in humans. Therefore, administration via diet should be preferred. The test compound(s) should be given in doses relevant to present or expected human exposures or resulting in the serum concentrations of phytoestrogens similar to humans. However, when the *in vivo* metabolites of phytoestrogens are the active compounds, the inter-individual variation in the bioavailability and metabolism may be difficult to standardize. In these cases, testing the metabolites instead of parent compounds need to be considered.

The great diversity and multiple mechanisms of action of phytoestrogens complicate interpretation of their health effects. Therefore, the understanding of health effects of dietary phytoestrogens is far from complete. Further development of *in vitro* assays suitable for measurement of estrogenicity in multiple cell-types is recommended for example, standardized *in vivo* tests for ER $\beta$  agonists are still lacking. Moreover, further development and evaluation of animal models is necessary to improve and facilitate extrapolation from *in vivo* animal data to human situation. In addition, further data on differences in bioavailability and metabolism of phytoestrogens between the test laboratory animal species and humans should be generated and compared.

In conclusion, further standardization and validation of the experimental settings used for testing potential health effects of dietary phytoestrogens are recommended in order to better understand both the potential benefits and risks associated with the long-term use of endocrine active dietary compounds such as phytoestrogens in humans.

## ACKNOWLEDGEMENTS

This work was supported by EU Phytohealth Thematic Network (QLK1-2002-2453).

## CONFLICT OF INTEREST

The authors have no conflict of interest directly relevant to the content of this paper.

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